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(57) Abstract

Factor VIII:C polypeptide analogs are provided that are native Factor VIII:C polypeptides that contain amino acid modifications at one or more amino acid residues adjacent an Arg residue, provided that the Arg residue is not at a Factor VIII:C activation site. Such modifications create one or more Arg-Pro or Pro-Arg linkages. Nucleic acid molecules encoding Factor VIII:C polypeptide analogs, vectors and host cells containing such nucleic acid molecules are also provided. Additional modifications include the creation of a tripeptide having the formula P_3-P_2-P_1, wherein P_3 is a residue selected from the group consisting of Phe, Glu, and Pro; P_2 is any amino acid residue except Ser and P2 is not Leu335 and is not Asn1720; and P_1 is Arg. Other modifications include substitutions at the non-activating Arg residues occurring at positions Arg336, Arg1719 and/or Arg1721. Further provided are analog complexes that contain at least two such analogs. Methods of producing the analogs, the analog complexes, nucleic acids encoding the same, vectors, and host cells are also provided as well as methods of using such compositions for prevention or treatment of active Factor VIII:C polypeptide deficiencies.

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NOVEL FACTOR VIII:C POLYPEPTIDE ANALOGS WITH ALTERED PROTEASE SITES

Field of the Invention

This invention relates to the discovery that an active Factor VIII:C polypeptide analog can be made that is modified at a site adjacent to an arginine ("Arg") residue, where the Arg is at a site other than at an activating site, creating an arginine-proline ("Arg-Pro") linkage or a proline-arginine ("Pro-Arg") linkage. This invention also relates to a Factor VIII:C polypeptide analog with a modification that comprises creation of a tripeptide having the formula P_3-P_2-P_1, wherein P_3 is a residue selected from the group consisting of Phe, Glu, and Pro; P_2 is any amino acid residue except Ser and P2 is not Leu335 and is not Asn1720; and P_1 is Arg. Additionally, the invention pertains to substitutions at the non-activating Arg residues occurring at positions Arg336, Arg1719 and/or Arg1721. This invention further relates to an analog complex comprising at least two such analogs, or one such analog and a native Factor VIII:C polypeptide, nucleic acid molecules encoding such analogs, vectors and host cells comprising the nucleic acid molecules, pharmaceutical compositions comprising the analogs or analog complexes, methods of making the analogs, nucleic acid molecules, vectors and host cells, and methods of prevention or treatment of active Factor VIII:C deficiency using the analogs, complexes, and/or nucleic acid molecules, vectors and host cells.

Background of the Invention

Hemophilia A is an X-chromosome-linked inherited bleeding diathesis that results from the

deficiency of an active blood clotting factor termed Factor VIII:C. The disease afflicts approximately 1 in 10,000 males. Factor VIII:C is a large glycoprotein that participates in the blood coagulation cascade that ultimately converts soluble fibrinogen to insoluble fibrin clot, effecting hemostasis.

The deduced primary amino acid sequence of human Factor VIII:C determined from the cloned cDNA indicates that Factor VIII:C is a heterodimer processed from a larger precursor polypeptide consisting of 2351 amino acids, referred to herein as the precursor or full-length Factor VIII:C molecule, of which the first 19 N-terminal residues comprise the signal sequence. Therefore, the mature Factor VIII:C molecule, starting with Ala1, which does not contain the signal peptide sequence, includes a sequence of 2332 amino acids. Amino acids from about 1 to about 1648 of the mature Factor VIII:C molecule give rise to "heavy chain" fragments with molecular weights ranging from approximately 90 kD to 200 kD. Amino acids from about 1649 to about 2331 of the mature Factor VIII:C molecule comprise a "light chain" with a molecular weight of approximately 80 kD ("the 80 kD subunit"). The heterodimeric mature Factor VIII:C molecule consists of the heavy and light chains associated by a metal ion bridge, and lacking amino acids 741-1648 (the B domain).

The mature Factor VIII:C molecule consists of a triplicated A domain of 330 amino acids, a unique B domain of 980 amino acids, and a duplicated C domain of 150 amino acids with the structure NH_2-A1-A2-B-A3-C1-C2-COOH. See, e.g., Kaufman, R. J., *Structure and Biology of Factor VIII*, in Part VI, Hemostasis and Thrombosis, pp. 1276-1284; and Pan et al., *Nature Structural Biology* (1995) *2*:740-744.

Factor VIII:C is known to be activated by plasma proteases such as thrombin. During activation, the mature Factor VIII:C polypeptide is cleaved to

generate heavy and light chain fragments that are further cleaved. For example, cleavage of the light chain after arginine residue 1689 ("Arg1689") yields a light chain fragment of about 73 kD ("the 73 kD fragment"), and cleavage of the heavy chain after arginine residue 372 ("Arg372") yields smaller heavy chain fragments of about 50 kD and 43 kD ("the 50 kD and 43 kD fragments," respectively), as described in Eaton *et al.* (1986), *Biochem. 25*: 505-512. At a minimum, the complex formed by the 50 kD and 73 kD polypeptides appears to be required for Factor VIII:C coagulant activity. Following activation, the heavy and light chain fragments of Factor VIII:C are inactivated by plasma proteases.

Patients suffering from hemophilia A are conventionally treated with purified or substantially purified Factor VIII:C. A difficulty in such treatment is the relatively short half-life of externally administered Factor VIII:C, lasting about 8 to 12 hours. This instability of Factor VIII:C derives in part from its susceptibility to proteolytic cleavage by plasma protease. Such plasma proteases, for example, thrombin, Factor Xa, and activated protein C ("APC"), inactivate Factor VIII:C by cleaving the molecule at multiple sites.

It would be advantageous, therefore, to produce Factor VIII:C polypeptides with improved properties.

Summary of the Invention

It is, therefore, an object of the present invention to provide a Factor VIII:C polypeptide analog that has improved properties.

In accordance, therewith, there is provided an active Factor VIII:C polypeptide analog that is substantially the same as a native Factor VIII:C polypeptide, except for modification at a site that is adjacent to a non-activating Arg residue, that is, adjacent to an Arg residue that is not at an activation site, such as Arg1689 or Arg372. The modification of the

present invention creates an Arg-Pro linkage or a Pro-Arg linkage. Such a modification can be achieved by one or more amino acid substitutions, additions or deletions.

In accordance with another object of the present invention, there is provided an analog as above where the non-activating Arg residue is at least one selected from the group consisting of amino acid residues 220, 226, 250, 279, 282, 336, 359, 562, 747, 776, 1310, 1313, 1645, 1648, 1719, and 1721, numbered with respect to the native Factor VIII:C polypeptide sequence, as depicted in Figures 1A-1F.

In accordance with another object of the present invention, there is provided an analog with a modification that comprises creation of a tripeptide having the formula P_3-P_2-P_1, wherein P_3 is a residue selected from the group consisting of Phe, Glu, and Pro; P_2 is any amino acid residue except Ser and P2 is not Leu335 and is not Asn1720; and P_1 is Arg.

In accordance with another object of the invention, there is provided an an active Factor VIII:C polypeptide analog comprising a native Factor VIII:C polypeptide that is modified at at least one non-activating Arg residue selected from the group consisting of Arg336, Arg1719 and Arg1721, wherein the modification comprises a substitution of any of amino acids Pro, Glu, Asp, Asn, Gln, Ser and Tyr for Arg336, a substitution of any of amino acids Pro, Glu, Asp, Asn, Gln, Ser and Tyr for Arg1719 and/or a substitution of any of amino acids Glu, Asp, Asn, Gln, Ser and Tyr for Arg1721, numbered with respect to the native Factor VIII:C polypeptide sequence.

In accordance with a further object of the present invention, there is provided an analog as above where the native Factor VIII:C polypeptide that is modified is selected from the group consisting of (a) a full-length Factor VIII:C molecule comprising a signal peptide and all A, B, and C domains; (b) a native Factor

VIII:C molecule comprising all A, B, and C domains and lacking a signal peptide; (c) a truncated Factor VIII:C molecule lacking a signal peptide and at least a portion of the B domain; (d) a cleaved Factor VIII:C molecule containing a light chain subunit of molecular weight of about 80 kD; (e) a cleaved Factor VIII:C molecule containing a heavy chain fragment of molecular weight in a range of about 90 kD to about 200 kD; (f) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of a molecular weight of about 90 kD; (g) a cleaved Factor VIII:C molecule containing a heavy chain fragment of molecular weight of about 50 kD, (h) a cleaved Factor VIII:C molecule containing a heavy chain fragment of molecular weight of about 43 kD; and (i) a cleaved Factor VIII:C molecule containing a light chain fragment of molecular weight of about 73 kD.

In accordance with a further object of the present invention, there is provided an active Factor VIII:C analog complex that contains either at least two Factor VIII:C polypeptide analogs as above, or a Factor VIII:C polypeptide analog and a Factor VIII:C polypeptide, together with a metal ion. For example, the analog complex herein can comprise two Factor VIII:C polypeptide analogs, and the two analogs can be selected from the group consisting of analogs of molecular weights of about (a) 80 kD and 90 kD; (b) 73 kD and 90 kD; (c) 80 kD and 50 kD; (d) 80 kD and 43 kD; (e) 73 kD and 50 kD; and (f) 73 kD and 43 kD.

In accordance with yet another object of the present invention, there is provided a method of producing a Factor VIII:C polypeptide analog as above by (a) providing a native Factor VIII:C polypeptide that contains an amino acid sequence, and (b) modifying at least one amino acid residue in the amino acid sequence to produce an analog as above.

In accordance with still another object of the present invention, there is provided a nucleic acid

present invention creates an Arg-Pro linkage or a Pro-Arg linkage. Such a modification can be achieved by one or more amino acid substitutions, additions or deletions.

In accordance with another object of the present invention, there is provided an analog as above where the non-activating Arg residue is at least one selected from the group consisting of amino acid residues 220, 226, 250, 279, 282, 336, 359, 562, 747, 776, 1310, 1313, 1645, 1648, 1719, and 1721, numbered with respect to the native Factor VIII:C polypeptide sequence, as depicted in Figures 1A-1F.

In accordance with another object of the present invention, there is provided an analog with a modification that comprises creation of a tripeptide having the formula P_3-P_2-P_1, wherein P_3 is a residue selected from the group consisting of Phe, Glu, and Pro; P_2 is any amino acid residue except Ser and P2 is not Leu335 and is not Asn1720; and P_1 is Arg.

In accordance with another object of the invention, there is provided an an active Factor VIII:C polypeptide analog comprising a native Factor VIII:C polypeptide that is modified at at least one non-activating Arg residue selected from the group consisting of Arg336, Arg1719 and Arg1721, wherein the modification comprises a substitution of any of amino acids Pro, Glu, Asp, Asn, Gln, Ser and Tyr for Arg336, a substitution of any of amino acids Pro, Glu, Asp, Asn, Gln, Ser and Tyr for Arg1719 and/or a substitution of any of amino acids Glu, Asp, Asn, Gln, Ser and Tyr for Arg1721, numbered with respect to the native Factor VIII:C polypeptide sequence.

In accordance with a further object of the present invention, there is provided an analog as above where the native Factor VIII:C polypeptide that is modified is selected from the group consisting of (a) a full-length Factor VIII:C molecule comprising a signal peptide and all A, B, and C domains; (b) a native Factor

composition that contains an active Factor VIII:C polypeptide analog or analog complex as above, and a pharmaceutically acceptable excipient.

Furthermore, there is also provided, in accordance with another object of the present invention, methods for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of (a) an active Factor VIII:C polypeptide analog as above, or (b) an active Factor VIII:C polypeptide analog complex as above, or (c) a nucleic acid molecule as above, or (d) a recombinant vector as above, or (e) a nucleic acid molecule as above together with and an active Factor VIII:C polypeptide analog, or (f) a recombinant vector as above together with an active Factor VIII:C polypeptide analog.

Further objects, features, and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description, while indicating the preferred embodiments of the invention, is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Figure

Figures 1A-1F depict the amino acid sequence of native Factor VIII:C.

Detailed Description of the Invention

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All of these publications and

applications, cited previously or below are hereby incorporated by reference.

The inventors herein have discovered that Factor VIII:C polypeptide analogs can be made that have improved properties. These analogs include one or more amino acid residues that are modified from the native structure. The modification can be at least one amino acid substitution, addition or deletion, at an amino acid residue adjacent to an Arg residue so as to generate, for example, an Arg-Pro linkage or a Pro-Arg linkage. Furthermore, the Factor VIII:C polypeptide analog can also include a modification that comprises creation of a tripeptide having the formula P_3-P_2-P_1, wherein P_3 is a residue selected from the group consisting of Phe, Glu, and Pro; P_2 is any amino acid residue except Ser and P2 is not Leu335 and is not Asn1720; and P_1 is Arg. The above modifications do not occur at a site of Factor VIII:C activation. Additionally, the non-activating Arg residues, Arg336 and Arg1719 can be substituted with amino acids Pro, Glu, Asp, Asn, Gln, Ser and Tyr and/or the non-activating Arg residue Arg1721 can be substituted with Glu, Asp, Asn, Gln, Ser and Tyr, to impart a Factor VIII:C analog with improved properties.

Definitions

The term "Factor VIII:C polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product. Thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid, including, for example, unnatural amino acids, polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring

and non-naturally occurring. A Factor VIII:C polypeptide includes but is not limited to, for example, the following Factor VIII:C polypeptides: (a) a full-length Factor VIII:C molecule comprising a signal peptide and all A, B, and C domains; (b) a mature Factor VIII:C molecule comprising all A, B, and C domains and lacking the signal peptide; (c) a truncated Factor VIII:C molecule lacking the signal peptide and at least a portion of the B domain; (d) a cleaved Factor VIII:C molecule comprising a light chain subunit of about 80 kD; (e) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of about 90 kD; (f) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of about 50 kD; (g) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of about 43 kD; and (h) a cleaved Factor VIII:C molecule comprising a light chain fragment of about 73 kD.

Factor VIII:C polypeptides also include muteins or derivatives of the polypeptides with conservative amino acid changes that do not alter the biological activity of the polypeptide from which the mutein or derivative is made. Such muteins or derivatives may have, for example, amino acid insertions, deletions, or substitutions in the relevant molecule that do not substantially affect its properties. For example, the mutein or derivative can include conservative amino acid substitutions, such as substitutions which preserve the general charge, hydrophobicity/hydrophilicity, and/or stearic bulk of the amino acid substituted, for example Gly/Ala; Val/Ile/Leu; Asp/Glu; Lys/Arg; Asn/Gln; and Phe/Trp/Tyr. The mutein or derivative should exhibit the same general structure as the native polypeptide, and may also include polypeptides having one or more peptide mimics or peptoids.

The term "active" in reference to the polypeptide analogs herein refers to biological activity, such as coagulation or pro-coagulation activity. Such

activity is measured by using standard assays for blood plasma samples, such as, for example, the Coatest assay or the activated partial thromboplastin time test (APTT). An "active" Factor VIII:C polypeptide analog will have at least about 50% of the coagulation or pro-coagulation activity displayed by the native molecule, preferably at least about 60% to 80%, and more preferably at least about 90% or more of the coagulation or procoagulation activity displayed by the native Factor VIII:C molecule.

A "nucleic acid molecule" as used herein, refers to either RNA or DNA or its complementary strands thereof, that contains a nucleotide sequence.

The term "regulatory element" refers to an expression control sequence that is conventionally used to effect expression of a gene. A regulatory element includes one or more components that affect transcription or translation, including transcription and translation signals. Such a sequence can be derived from a natural source or synthetically made, as in hybrid promoters and includes, for example, one or more of a promoter sequence, an enhancer sequence, a combination promoter/enhancer sequence, an upstream activation sequence, a downstream termination sequence, a polyadenylation sequence, an optimal 5' leader sequence to optimize initiation of translation, and a Shine-Dalgarno sequence. The expression control sequence that is appropriate for expression of the present polypeptide differs depending upon the host system in which the polypeptide is to be expressed. For example, in prokaryotes, such a sequence can include one or more of a promoter sequence, a ribosomal binding site, and a transcription termination sequence. In eukaryotes, for example, such a sequence can include one or more of a promoter sequence, and a transcription termination sequence. If any component that is necessary for transcription or translation is lacking in the nucleic acid molecule of the present invention, such a component

can be supplied by a vector. Regulatory elements suitable for use herein may be derived from a prokaryotic source, an eukaryotic source, a virus or viral vector or from a linear or circular plasmid.

The term "regulatory control" refers to control of expression of a polynucleotide sequence by a regulatory element to which the polynucleotide sequence is operably linked. The nature of such regulatory control differs depending upon the host organism. In prokaryotes, such regulatory control is effected by regulatory sequences which generally include, for example, a promoter, and/or a transcription termination sequence. In eukaryotes, generally, such regulatory sequences include, for example, a promoter and/or a transcription termination sequence. Additionally, other components which control of expression, for example, a signal peptide sequence or a secretory leader sequence for secretion of the polypeptide, and a terminator for transcriptional termination, may be attached thereto to facilitate regulatory control of expression.

By "therapeutically effective amount" is meant an amount of analog that will improve the blood coagulation properties as compared to coagulation in the absence of the analog. A "therapeutically effective amount" will fall within a relatively broad range that can be determined through routine trials. The activity of the Factor VIII:C analogs of the invention may be determined by means known in the art, for example, by using the commercially available Coatest assay. Preferably, the effective amount is sufficient to bring about prevention of further deterioration or treatment to improve coagulation, that is, to enhance coagulation properties such that hemostasis is achieved. The exact amount necessary will vary depending on the subject being treated; the age and general condition of the individual to be treated; the functionality of the endogenous Factor VIII:C gene present in the individual; and the mode of

administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. For example, depending on the severity of active Factor VIII:C polypeptide deficiency, up to about 1000 to about 3000 U of Factor VIII:C polypeptide analog can be given to an average person such as a 70 kg male patient. Alternatively, sufficient Factor VIII:C polypeptide analog or analog complex can be given to establish a plasma level of about 0.5 to about 2 U/ml of Factor VIII:C analog or combination analog and native polypeptide. See U.S. Patent Nos. 3,631,018; 3,652,530, and 4,069,216 for methods of administration and amounts.

The term "pharmaceutically acceptable excipient" refers to an excipient for administration of a therapeutic agent, *in vivo*, and refers to any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Suitable carriers may also be present and are generally large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack

Pub. Co., N.J. 1991). Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared.

The present invention provides Factor VIII:C polypeptide analogs with improved properties. The analogs include Pro-Arg bonds or Arg-Pro bonds at non-activating Arg residues. Preferably, the modification will not introduce an Arg-Ala, Arg-Met, Arg-Gly, Arg-Ser or Arg-Thr bond at the modified site.

Additionally, the modification can comprise creation of a tripeptide having the formula P_3-P_2-P_1, wherein P_3 is a residue selected from the group consisting of Phe, Glu, and Pro; P_2 is any amino acid residue except Ser and P2 is not Leu335 and is not Asn1720; and P_1 is Arg.

Thus, when the non-activating Arg residue is Arg220, representative modifications can comprise an insertion of at least one Pro residue between Asp219 and Arg 220; an insertion of at least one Pro residue between Arg220 and Asp221; a deletion comprising residues Asp221, Ala222, Ala223, Ser224, Ala225, Arg226, Ala227, and Trp228; an insertion of at least one Phe residue between Gln218 and Asp219; an insertion of at least one Glu residue between Gln218 and Asp219; an insertion of at least one Pro residue between Gln218 and Asp219; a deletion comprising residues Thr212, Lys213, Asn214, Ser215, Leu216, Met217, and Gln218; a substitution of at least one Phe residue at Gln218; a substitution of at least one Glu residue at Gln218; and/or a substitution of at least one Pro residue at Gln218.

When the non-activating Arg residue is Arg226, representative modifications can comprise an insertion of at least one Pro residue between Ala225 and Arg226; an insertion of at least one Pro residue between Arg226 and Ala227; a deletion comprising residues Ala227 and Trp228;

a deletion comprising residues Ala227 and Trp228 and insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Ala227, Trp228, Pro229, and Lys230, and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Phe residue between Ser224 and Ala225; an insertion of at least one Glu residue between Ser224 and Ala225; an insertion of at least one Pro residue between Ser224 and Ala225; (d) a substitution of at least one Phe residue at Ser224; a substitution of at least one Glu residue at Ser224; and/or a substitution of at least one Pro residue at Ser224.

When the non-activating Arg residue is Arg250, representative modifications can comprise an insertion of at least one Pro residue between His249 and Arg250; an insertion of at least one Pro residue between Arg250 and Lys251; a deletion comprising residues Lys251 and Ser252 and insertion of at least one Pro residue to replace the deleted residues; a substitution of Lys251 with at least one Pro residue; a deletion comprising residues Gly244, Leu245, Ile246, Gly247, Cys248, and His249; a deletion comprising residues Arg240, Ser241, Leu242, Pro243, Gly244, Leu245, Ile246, Gly247, Cys248, and His249 and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Phe residue between Cys248 and His249; an insertion of at least one Glu residue between Cys248 and His249; an insertion of at least one Pro residue between Cys248 and His249; a deletion comprising residues Gly244, Leu245, Ile246, Gly247, and Cys248; a substitution of at least one Phe residue at Cys248; a substitution of at least one Glu residue at Cys248; and/or a substitution of at least one Pro residue at Cys248.

When the non-activating Arg residue is Arg279, representative modifications can comprise an insertion of at least one Pro residue between Val278 and Arg279; an insertion of at least one Pro residue between Arg279 and

Asn280; a deletion comprising residues Asn280, His281, and Arg282 and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising Asn280, His281, Arg282, Gln283, Ala284, and Ser285, and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Phe residue between Leu277 and Val278; an insertion of at least one Glu residue between Leu277 and Val278; an insertion of at least one Pro residue between Leu277 and Val278; a deletion comprising residue Leu277; a deletion comprising residue Val278; a deletion comprising residues Gly273, His274, Thr275, Phe276, and Leu277; a deletion comprising residues Leu271, Glu272, Gly273, His274, Thr275, Phe276, and Leu277; a substitution of at least one Phe residue at Leu277; a substitution of at least one Glu residue at Leu277; and/or a substitution of at least one Pro residue at Leu277.

When the non-activating Arg residue is Arg282, representative modifications can comprise an insertion of at least one Pro residue between His281 and Arg282; an insertion of at least one Pro residue between Arg282 and Gln283; a deletion comprising residues Arg279, Asn280, and His281 and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Gln283, Ala284, and Ser285, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Gln283, Ala284, Ser285, Leu286, Glu287, Ile288, and Ser289; an insertion of at least one Phe residue between Asn280 and His281; an insertion of at least one Glu residue between Asn280 and His281; an insertion of at least one Pro residue between Asn280 and His281; a deletion comprising residues Leu277, Val278, Arg279, and Asn280; a deletion comprising residues Gly273, His274, Thr275, Phe276, Leu277, Val278, Arg279, and Asn280; a substitution of at least one Phe residue at Asn280; a substitution of at least one Glu

residue at Asn280; and/or a substitution of at least one Pro residue at Asn280.

When the non-activating Arg residue is Arg336, representative modifications can comprise an insertion of at least one Pro residue between Leu335 and Arg336; a deletion comprising residues Gln334, and Leu335; an insertion of at least one Pro residue between Arg336 and Met337; a deletion comprising residues Met337, and Lys338 and insertion of at least one Pro residue in place of the deleted residues; a deletion comprising residue Leu335 and an insertion of at least one Phe residue between Pro333 and Gln334; a deletion comprising residue Leu335 and an insertion of at least one Glu residue between Pro333 and Gln334; a deletion comprising residue Leu335 and an insertion of at least one Pro residue between Pro333 and Gln334; a deletion comprising residue Leu335; a deletion comprising residues Gln334, and Leu335; a deletion comprising residues Pro333, Gln334, and Leu335; a deletion comprising residues Glu332, Pro333, Gln334, and Leu335; a deletion comprising residue Leu335 and a substitution of at least one Phe residue at Pro333; and/or a deletion comprising residue Leu335 and a substitution of at least one Glu residue at Pro333.

When the non-activating Arg residue is Arg359, representative modifications can comprise an insertion of at least one Pro residue between Val358 and Arg359; an insertion of at least one Pro residue between Arg359 and Phe360; a deletion comprising residues Phe360, Asp361, Asp362, Asp363, Asn364, and Ser365; a deletion comprising residues Phe360, Asp361, Asp362, Asp363, Asn364, Ser365, Pro366, and Ser367, and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Phe residue between Val357 and Val358; an insertion of at least one Glu residue between Val357 and Val358; an insertion of at least one Pro residue between Val357 and Val358; a deletion comprising residues Met355, Asp356, and Val357; a substitution of at least one Phe

residue at Val357; a substitution of at least one Glu residue at Val357; and/or a substitution of at least one Pro residue at Val357.

When the non-activating Arg residue is Arg562, representative modifications can comprise an insertion of at least one Pro residue between Gln561 and Arg562; an insertion of at least one Pro residue between Arg562 and Gly563; a deletion comprising residues Ser558, Val559, Asp560, Gln561, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Lys556, Glu557, Ser558, Val559, Asp560, Gln561, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Gly563, Asn564, Gln565, Ile566, Met567, and Ser568, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Gly563, Asn564, Gln565, Ile566, Met567, Ser568, Asp569, Lys570, and Arg571, and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Phe residue between Asp560 and Gln561; an insertion of at least one Glu residue between Asp560 and Gln561; an insertion of at least one Pro residue between Asp560 and Gln561; a deletion comprising residues Ser558, Val559, and Asp560; a substitution of at least one Phe residue at Asp560; a substitution of at least one Glu residue at Asp560; and/or a substitution of at least one Pro residue at Asp560.

When the non-activating Arg residue is Arg747, representative modifications can comprise an insertion of at least one Pro residue between Ser746 and Arg747; a deletion comprising residue Ser746 and an insertion of at least one Pro residue to replace the deleted residue; a deletion comprising residue His748; a deletion comprising residue His748 and an insertion of at least one Pro residue to replace the deleted residue; an insertion of at least one Pro residue between Arg747 and His748; a

deletion comprising residues His748, Pro749, and Ser750 and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising Ser743, Gln744, Asn745, Ser746; a deletion comprising His748, Pro749, Ser750, Thr751, Arg752, Gln753, and Lys754, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residue Ser746 and an insertion of at least one Phe residue between Gln744 and Asn745; a deletion comprising residue Ser746 and an insertion of at least one Glu residue between Gln744 and Asn745; a deletion comprising residue Ser746 and an insertion of at least one Pro residue between Gln744 and Asn745; a deletion comprising residue Ser746 and a substitution of at least one Phe residue at Gln744; a deletion comprising residue Ser746 and a substitution of at least one Glu residue at Gln744; and/or a deletion comprising residue Ser746 and a substitution of at least one Pro residue at Gln744.

When the non-activating Arg residue is Arg776, representative modifications can comprise an insertion of at least one Pro residue between His775 and Arg776; an insertion of at least one Pro residue between Arg776 and Thr777; a deletion comprising residue Thr777; a deletion comprising residue Thr777 and an insertion of at least one Pro residue to replace the deleted residue; a deletion comprising residues Trp772, Phe773, Ala774, and His775; a deletion comprising residues Trp772, Phe773, Ala774, and His775, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Lys768, Thr769, Asp770, Pro771, Trp772, Phe773, Ala774, and His775, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Thr777, Pro778, met779, Pro780, and Lys781, and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Phe residue between Ala774 and His775; an insertion of at least one Glu residue between Ala774 and

His775; an insertion of at least one Pro residue between Ala774 and His775; a deletion comprising residue Ala774; a deletion comprising residues Trp772, Phe773 and Ala774; a deletion comprising residues Lys768, Thr769, Asp770, Pro771, Trp772, and Phe773; a substitution of at least one Pheresidue at Ala774; a substitution of at least one Glu residue at Ala774; and/or a substitution of at least one Pro residue at Ala774.

When the non-activating Arg residue is Arg1310, representative modifications can comprise an insertion of at least one Pro residue between Gln1309 and Arg1310; an insertion of at least one Pro residue between Arg1310 and Ser1311; a deletion comprising residue Ser1311 and an insertion of at least one Pro residue to replace the residue; a deletion comprising residues Ser1311, Lys1312, and Arg1313 and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Ser1311, Lys1312, Arg1313, Ala1314, Leu1315, and Lys1316, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Ser1311, Lys1312, Arg1313, Ala1314, Leu1315, Lys1316, Gln1317, Phe1318, Arg1319, and Leu1320; a deletion comprising residues Ser1311, Lys1312, Arg1313, Ala1314, Leu1315, Lys1316, Gln1317, Phe1318, Arg1319, and Leu1320, and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Phe residue between Thr1308 and Gln1309; an insertion of at least one Glu residue between Thr1308 and Gln1309; an insertion of at least one Pro residue between Thr1308 and Gln1309; a deletion comprising residues Val1307, and Thr1308; a substitution of at least one Phe residue at Thr1308; a substitution of at least one Glu residue at Thr1308; and/or a substitution of at least one Pro residue at Thr1308.

When the non-activating Arg residue is Arg1313, representative modifications can comprise an insertion of at least one Pro residue between Lys1312 and Arg1313; a

deletion comprising residue Lys1312 and an insertion of at least one Pro residue to replace the deleted residue; a deletion comprising residues Arg1310, Ser1311, and Lys1312, and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Pro residue between Arg1313 and Ala1314; a deletion comprising residues Ala1314, Leu1315, and Lys1316, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Ala1314, Leu1315, Lys1316, Gln1317, Phe1318, and Arg1319 and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Ala1314, Leu1315, Lys1316, Gln1317, Phe1318, Arg1319, and Leu1320; an insertion of at least one Phe residue between Ser1311 and Lys1312; an insertion of at least one Glu residue between Ser1311 and Lys1312; an insertion of at least one Pro residue between Ser1311 and Lys1312; a deletion comprising residues Val1307, Thr1308, Gln1309, Arg1310, and Ser1311; a substitution of at least one Phe residue at Ser1311; a substitution of at least one Glu residue at Ser 1311; and/or a substitution of at least one Pro residue at Ser 1311.

When the non-activating Arg residue is Arg1645, representative modifications can comprise a deletion comprising residues Val1642, Leu1643, and Lys1644; a deleteion comprising residue Lys1644 and an insertion of at least one Pro residue to replace the deleted residue; an insertion of at least one Pro residue between Arg1645 and Lys1644; an insertion of at least one Pro residue between Arg1645 and His1646; a deletion comprising residues His1646, Gln1647, and Arg1648 and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues His1646, Gln1647, Arg1648, Glu1649, Ile1650, Thr1651, and Arg1652, and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Phe residue between Leu1643 and Lys1644; an insertion of at

least one Glu residue between Leu1643 and Lys1644; an insertion of at least one Pro residue between Leu1643 and Lys1644; a deletion comprising residues Val1642, and Leu1643; a deletion comprising residues Pro1641, Val1642, and Leu1643; a substitution of at least one Phe residue at Leu1643; a substitution of at least one Glu residue at Leu1643; and/or a substitution of at least one Pro residue at Leu1643.

When the non-activating Arg residue is Arg1648, representative modifications include a deletion comprising residues Val1642, Leu1643, Lys1644, Arg1645, His1646, and Gln1647; an insertion of at least one Pro residue between Gln1647 and Arg1648; a deletion comprising residues Lys1644, Arg1645, His1646, and Gln1647 and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Glu1649, Ile1650, Thr1651, and Arg1652 and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Pro residue between Arg1648 and Glu1649; an insertion of at least one Phe residue between His1646 and Gln1647; an insertion of at least one Glu residue between His1646 and Gln1647; an insertion of at least one Pro residue between His1646 and Gln1647; a deletion comprising residues Val1642, Leu1643, Lys1644, Arg1645, and His1646; a deletion comprising residues Pro1641, Val1642, Leu1643, Lys1644, Arg1645, and His1646; a substitution of at least one Phe residue at His1646; (g) a substitution of at least one Glu residue at His1646; and/or a substitution of at least one Pro residue at His1646.

When the non-activating Arg residue is Arg1719, representative modifications a deletion comprising residues His1716, Val1717, and Leu1718; an insertion of at least one Pro residue between Leu1718 and Arg1719; a deletion comprising residues Asn1720, and Arg1721 and an insertion of at least one Pro residue to replace the deleted residues; an insertion of at least one Pro

residue between Arg1719 and Asn1720; an insertion of at least one Phe residue between Val1717 and Leu1718; an insertion of at least one Glu residue between Val1717 and Leu1718; an insertion of at least one Pro residue between Val1717 and Leu1718; a deletion comprising residues His1716, and Val1717; a substitution of at least one Phe residue at Val1717; a substitution of at least one Glu residue at Val1717; and/or a substitution of at least one Pro residue at Val1717.

When the non-activating Arg residue is Arg1721, representative modifications include a deletion comprising residues His1716, Val1717, Leu1718, Arg1719, and Asn1720; an insertion of at least one Pro residue between Asn1720 and Arg1721; an insertion of at least one Pro residue between Arg1721 and Ala1722; a deletion comprising residues Ala1722, Gln1723, and Ser1724 and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residues Ala1722, Gln1723, Ser1724, Gly1725, Ser1726, and Val1727; a deletion comprising residues Ala1722, Gln1723, Ser1724, Gly1725, and Ser1726, and an insertion of at least one Pro residue to replace the deleted residues; a deletion comprising residue Asn1720 and an insertion of at least one Phe residue between Leu1718 and Arg1719; a deletion comprising residue Asn1720 and an insertion of at least one Glu residue between Leu1718 and Arg1719; a deletion comprising residue Asn1720 and an insertion of at least one Pro residue between Leu1718 and Arg1719; a deletion comprising residues Val1717, Leu1718, Arg1719, and Asn1720; a deletion comprising residue Asn1720 and a substitution of at least one Phe residue at Leu1718; a deletion comprising residue Asn1720 and a substitution of at least one Glu residue at Leu1718; and/or a deletion comprising residue Asn1720 and a substitution of at least one Pro residue at Leu1718.

Other polypeptide analogs contemplated by the present invention are those analogs including

substitutions of the non-activating Arg residues found at positions Arg336, Arg1719 and Arg1721, numbered with respect to the native Factor VIII:C polypeptide sequence. For example, based on the model of Factor VIII:C described in Pan et al., *Nature Structural Biology* (1995) *2*:740-744, any of amino acids Pro, Glu, Asp, Asn, Gln, Ser and Tyr can be substituted at position Arg336 and/or position Arg1719, to generate a Factor VIII:C polypeptide analog with improved properties. Similarly, any of amino acids Glu, Asp, Asn, Gln, Ser and Tyr can be substituted at position Arg1721. Preferably, the Factor VIII:C polypeptide analog of this embodiment will include a substitution of Arg336 with Pro, a substitution of Arg 1719 with Pro and/or a substitution of Arg1721 with Glu.

Nucleic acid molecules encoding the present Factor VIII:C polypeptide analogs can be made by modifying the native nucleic acid sequences that encode the Factor VIII:C polypeptide or cDNA sequences that encode the Factor VIII:C polypeptides. Such modification can be done by conventional techniques such as site-directed mutagenesis. For example, the M13 method for site directed mutagenesis is known, as described in Zoller and Smith, *Nucleic Acids Res.* (1982) 10: 6487-6500, *Methods Enzymol.* (1983) 100: 468-500, and *DNA* (1984) 3: 479-488, using single stranded DNA, and the method of Morinaga *et al. Bio/technol.* 636-639 (July 1984), using heteroduplexed DNA. According to the method of the invention, by site-directed mutagenesis, one or more of the codons encoding a residue adjacent to an arginine, preferably, at the carboxy side, can be mutated by substitution, deletion or addition, to a codon encoding proline, thereby creating polypeptides with either Pro-Arg bonds or Arg-Pro bonds where none existed before, provided that the modification does not affect an activation cleavage site, such as Arg372 and Arg740. The codons encoding proline include CCU, CCT, CCG, CCA, and CCC. A description of a protocol suitable for use herein

for mutagenesis of specific sites of a Factor VIII:C expression plasmid can be found in WO 87/07144.

The nucleic acid molecules of the present invention can also be made synthetically by piecing together nucleic acid molecules encoding heavy and light chain fragments derived from cDNA clones or genomic clones containing Factor VIII:C coding sequences, preferably cDNA clones, using known linker sequences. Alternatively, the entire sequence or portions of nucleic acid sequences encoding analogs described above may be prepared by synthetic methods (e.g. using DNA synthesis machines). Once made, the nucleic acid molecules can be inserted in vectors for production of recombinant vectors for transcription and translation of the nucleic acid molecules.

One skilled in the art of DNA cloning and in possession of the DNA encoding native Factor VIII:C polypeptide will be able to prepare suitable DNA molecules for production of the present analogs using known cloning procedures (e.g. restriction enzyme digestion, exonuclease digestion, ligation, and other appropriate procedures) outlined in any of the following: Sambrook, *et al*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd ed. (Cold Spring Harbor Laboratory Press, 1989); DNA CLONING, Vol. I and II, D.N. Glover ed. (IRL Press, 1985); OLIGONUCLEOTIDE SYNTHESIS, M.J. Gait ed. (IRL Press, 1984); NUCLEIC ACID HYBRIDIZATION, B.D. Hames & S.J. Higgins eds. (IRL Press, 1984); TRANSCRIPTION AND TRANSLATION, B.D. Hames & S.J. Higgins eds., (IRL Press, 1984); ANIMAL CELL CULTURE, R.I. Freshney ed. (IRL Press, 1986); IMMOBILIZED CELLS AND ENZYMES, K. Mosbach (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING, Wiley (1984); the series, METHODS IN ENZYMOLOGY, Academic Press, Inc.; GENE TRANSFER VECTORS FOR MAMMALIAN CELLS, J.H. Miller and M.P. Calos eds. (Cold Spring Harbor Laboratory, 1987); METHODS IN ENZYMOLOGY, Vol. 154 and 155, Wu and Grossman, eds., and Wu, ed., respectively

(Academic Press, 1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY, R.J. Mayer and J.H. Walker, eds. (Academic Press London, Harcourt Brace U.S., 1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, 2nd ed. (Springer-Verlag, N.Y. (1987), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, Vol. I-IV, D.M. Weir *et al*, (Blackwell Scientific Publications, 1986); Kitts *et al*, *Biotechniques,* (1993), *14*:810-817; Munemitsu *et al*, *Mol. Cell. Biol.*, (1990) *10*:5977-5982. Finally, a preferred method of preparing nucleic acid molecules encoding the described analogs is by use of PCR techniques, especially overlapping PCR, as described in PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, Innis, Gelfand, Sninsky, and White (eds.) (Academic Press, 1990).

A vector suitable for use herein for the production of a recombinant vector comprises a nucleic acid sequence with one or more restriction enzyme recognition sites into which the present nucleic acid molecule of the invention can be inserted. This vector also typically contains a selection marker for detection of the presence of the vector in the host cell. The vector can also provide, if desired, one or more regulatory elements or control sequences for expression of the nucleic acid molecule. For example, the present vector can be derived from a plasmid, a virus, a cosmid, or a bacteriophage. This vector is typically capable of behaving as an autonomous unit of replication when introduced into a host cell. Moreover, the vector may be one that is capable of episomal existence or of integration into the host cell genome. A wide variety of replication systems are available, typically derived from viruses that infect mammalian host cells. Illustrative replication systems include the replication systems from Simian virus 40, adenovirus, bovine papilloma virus, polyoma virus, Epstein Barr virus, and the like. Thus, the nucleic acid molecule of the present invention can be inserted at an appropriate restriction site in the vector

so as to be placed under the control of one or more regulatory elements in the vector to form a recombinant vector that can be used for transfection or transformation of a host cell.

The host cells of the invention can be, for example, prokaryotic or eukaryotic host cells, including bacterial, yeast, insect and mammalian expression systems. Preferably the analogs of the present invention are expressed in mammalian host cell systems.

The regulatory elements to be used in the vector depend on the host system that is to be utilized. For example, a prokaryotic host cell can be used for amplification of the nucleic acid molecule of the present invention, while an eukaryotic host cell can be used for expression of the Factor VIII:C polypeptide analogs.

The expression cassettes are introduced into the host cell by conventional methods, depending on the expression system used, as described further below. Where viruses are involved, transfection or transduction may be employed. The particular manner in which the host cell is transformed is not critical to this invention, depending substantially upon whether the expression cassettes are joined to a replication system and the nature of the replication system and associated genes.

Coexpression of more than one Factor VIII:C polypeptide analog may be desired. For example, it may be desirable to express the light and heavy chains using separate constructs. In this regard, either or both of the light and heavy chains may include modifications as described above. "Coexpression" as used herein refers to the expression of two or more Factor VIII:C polypeptides in a single host cell. Thus, for example, the expression of the 90 kD species and the 80 kD species in a single host cell, would constitute "coexpression" as used herein. The polynucleotides encoding for the polypeptides can be harbored in a single vector, either under the control of the same regulatory elements or

under the control of separate elements. Thus, the production of a fusion protein including active portions of the two or more Factor VIII:C polypeptides would be considered "coexpressed" for purposes of the present definition as would the expression of two genes as a dicistronic construct employing an internal ribosome entry site. Similarly, proteins expressed from the same vector but driven by separate regulatory elements, would also be considered "coexpressed." The term also refers to the expression of two or more proteins from separate constructs. Thus, the expression of proteins encoded from genes present on separate vectors in a host cell would also be considered "coexpression" for purposes of the present invention.

The transformed/transfected cells are then grown in an appropriate nutrient medium. If separate constructs encoding heavy and light chain analogs have been used for coexpression, the product can be obtained as a complex of the two Factor VIII:C chains, so that the media or cell lysate may be isolated and the Factor VIII:C active complex extracted and purified. Similarly, the full-length molecule can be isolated and treated under complex-forming conditions, e.g., with the addition of calcium and the appropriate enzymes, to form the active complex. Various means are available for extraction and purification, such as affinity chromatography, ion exchange chromatography, hydrophobic chromatography, electrophoresis, solvent-solvent extraction, selective precipitation, and the like. The particular manner in which the product is isolated is not critical to this invention, and is selected to minimize denaturation or inactivation and maximize the isolation of a high-purity active product.

Expression in Bacterial Cells

Bacterial expression systems can be used to produce the subject Factor VIII:C polypeptide analogs and nucleic acid sequences encoding the analogs. Control elements for use in bacterial systems include promoters, optionally containing operator sequences, and ribosome binding sites. Useful promoters include sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*), the β-lactamase (*bla*) promoter system, bacteriophage λPL, and T7. In addition, synthetic promoters can be used, such as the *tac* promoter. The β-lactamase and lactose promoter systems are described in Chang *et al.*, *Nature* (1978) *275*: 615, and Goeddel *et al.*, *Nature* (1979) *281*: 544; the alkaline phosphatase, tryptophan (trp) promoter system are described in Goeddel *et al.*, *Nucleic Acids Res.* (1980) *8*: 4057 and EP 36,776 and hybrid promoters such as the *tac* promoter is described in U.S. Patent No. 4,551,433 and deBoer *et al.*, *Proc. Natl. Acad. Sci. USA* (1983) *80*: 21-25. However, other known bacterial promoters useful for expression of eukaryotic proteins are also suitable. A person skilled in the art would be able to operably ligate such promoters to the present Factor VIII:C polypeptide analog coding sequences, for example, as described in Siebenlist *et al.*, *Cell* (1980) *20*: 269, using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (SD) sequence operably linked to the DNA encoding the Factor VIII:C analog polypeptide. For prokaryotic host cells that do not recognize and process the native polypeptide signal sequence, the signal sequence can be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, Ipp, or heat stable enterotoxin II

leaders. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria.

The foregoing systems are particularly compatible with *Escherichia coli*. However, numerous other systems for use in bacterial hosts including Gram-negative or Gram-positive organisms such as *Bacillus spp.*, *Streptococcus spp.*, *Streptomyces spp.*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*, among others. Methods for introducing exogenous DNA into these hosts typically include the use of $CaCl_2$ or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation, nuclear injection, or protoplast fusion as described generally in Sambrook *et al.* (1989), cited above. These examples are illustrative rather than limiting. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

Prokaryotic cells used to produce the Factor VIII:C analog polypeptides of this invention are cultured in suitable media, as described generally in Sambrook *et al.*, cited above.

Expression in Yeast Cells

Yeast expression systems can also be used to produce the subject Factor VIII:C polypeptide analogs and nucleic acid sequences encoding the analogs. Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors have been developed for, among others, the following yeasts: *Saccharomyces cerevisiae* ,as described in Hinnen *et al.*, *Proc. Natl. Acad. Sci. USA* (1978) *75*: 1929; Ito *et al.*, *J. Bacteriol.* (1983) *153*: 163; *Candida albicans* as described in Kurtz *et al.*, *Mol. Cell. Biol.*

(1986) *6*: 142; *Candida maltosa*, as described in Kunze *et al.*, *J. Basic Microbiol.* (1985) *25*: 141; *Hansenula polymorpha*, as described in Gleeson *et al.*, *J. Gen. Microbiol.* (1986) *132*: 3459 and Roggenkamp *et al.*, *Mol. Gen. Genet.* (1986) *202* :302); *Kluyveromyces fragilis*, as described in Das *et al.*, *J. Bacteriol.* (1984) *158*: 1165; *Kluyveromyces lactis*, as described in De Louvencourt *et al.*, *J. Bacteriol.* (1983) *154*: 737 and Van den Berg *et al.*, *Bio/Technology* (1990) *8*: 135; *Pichia guillerimondii*, as described in Kunze *et al.*, *J. Basic Microbiol.* (1985) *25*: 141; *Pichia pastoris*, as described in Cregg *et al.*, *Mol. Cell. Biol.* (1985) *5*: 3376 and U.S. Patent Nos. 4,837,148 and 4,929,555; *Schizosaccharomyces pombe*, as described in Beach and Nurse, *Nature* (1981) *300*: 706; and *Yarrowia lipolytica*, as described in Davidow *et al.*, *Curr. Genet.* (1985) *10*: 380 and Gaillardin *et al.*, *Curr. Genet.* (1985) *10*: 49, *Aspergillus* hosts such as *A. nidulans*, as described in Ballance *et al.*, *Biochem. Biophys. Res. Commun.* (1983) *112*: 284-289; Tilburn *et al.*, *Gene* (1983) *26*: 205-221 and Yelton *et al.*, *Proc. Natl. Acad. Sci. USA* (1984) *81*: 1470-1474, and *A. niger*, as described in Kelly and Hynes, *EMBO J.* (1985) *4*: 475479; *Trichoderma reesia*, as described in EP 244,234, and filamentous fungi such as, e.g, *Neurospora*, *Penicillium*, *Tolypocladium*, as described in WO 91/00357.

Control sequences for yeast vectors are known and include promoter regions from genes such as alcohol dehydrogenase (ADH), as described in EP 284,044, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK), as described in EP 329,203. The yeast *PHO5* gene, encoding acid phosphatase, also provides useful promoter sequences, as described in Myanohara *et al.*, *Proc. Natl. Acad. Sci. USA* (1983) *80*:1. Other suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate

kinase, as described in Hitzeman *et al.*, *J. Biol. Chem.* (1980) *255*: 2073, or other glycolytic enzymes, such as pyruvate decarboxylase, triosephosphate isomerase, and phosphoglucose isomerase, as described in Hess *et al.*, *J. Adv. Enzyme Reg.* (1968) 7: 149 and Holland *et al.*, *Biochemistry* (1978) *17*: 4900. Inducible yeast promoters having the additional advantage of transcription controlled by growth conditions, include from the list above and others the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EP 073,657. Yeast enhancers also are advantageously used with yeast promoters. In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, upstream activating sequences (UAS) of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region, as described in U.S. Patent Nos. 4,876,197 and 4,880,734. Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the *ADH2*, *GAL4*, *GAL10*, or *PHO5* genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as *GAP* or *PyK*, as described in EP 164,556. Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription.

Other control elements which may be included in the yeast expression vectors are terminators, for example, from *GAPDH* and from the enolase gene, as

described in Holland *et al.*, *J. Biol. Chem.* (1981) *256*: 1385, and leader sequences which encode signal sequences for secretion. DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene as described in EP 012,873 and JP 62,096,086 and the α-factor gene, as described in U.S. Patent Nos. 4,588,684, 4,546,083 and 4,870,008; EP 324,274; and WO 89/02463. Alternatively, leaders of non-yeast origin, such as an interferon leader, also provide for secretion in yeast, as described in EP 060,057.

Methods of introducing exogenous DNA into yeast hosts are well known in the art, and typically include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations. Transformations into yeast can be carried out according to the method described in Van Solingen *et al.*, *J. Bact.* (1977) *130*: 946 and Hsiao *et al.*, *Proc. Natl. Acad. Sci. USA* (1979) *76*: 3829. However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used as described generally in Sambrook *et al.*, cited above.

For yeast secretion the native polypeptide signal sequence may be substituted by the yeast invertase, α-factor, or acid phosphatase leaders. The origin of replication from the 2μ plasmid origin is suitable for yeast. A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid described in Kingsman *et al.*, *Gene* (1979) 7: 141 or Tschemper *et al.*, *Gene* (1980) *10*: 157. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* Gene.

For intracellular production of the present polypeptides in yeast, a sequence encoding a yeast protein can be linked to a coding sequence of a Factor

VIII:C polypeptide analog to produce a fusion protein that can be cleaved intracellularly by the yeast cells upon expression. An example, of such a yeast leader sequence is the yeast ubiquitin gene.

Expression in Insect Cells

Insect expression systems can be used to produce the Factor VIII:C polypeptide analogs and nucleic acid sequences encoding the analogs. For example, baculovirus expression vectors (BEVs) are recombinant insect viruses in which the coding sequence for a foreign gene to be expressed is inserted behind a baculovirus promoter in place of a viral gene, e.g., polyhedrin, as described in Smith and Summers, U.S. Pat. No., 4,745,051.

An expression construct herein includes a DNA vector useful as an intermediate for the infection or transformation of an insect cell system, the vector generally containing DNA coding for a baculovirus transcriptional promoter, optionally but preferably, followed downstream by an insect signal DNA sequence capable of directing secretion of a desired protein, and a site for insertion of the foreign gene encoding the foreign protein, the signal DNA sequence and the foreign gene being placed under the transcriptional control of a baculovirus promoter, the foreign gene herein being the coding sequence of a Factor VIII:C polypeptide analog of this invention.

The promoter for use herein can be a baculovirus transcriptional promoter region derived from any of the over 500 baculoviruses generally infecting insects, such as, for example, the Orders Lepidoptera, Diptera, Orthoptera, Coleoptera and Hymenoptera including, for example, but not limited to the viral DNAs *of Autographo californica* MNPV, *Bombyx mori* NPV, *rrichoplusia ni* MNPV, *Rachlplusia ou* MNPV or *Galleria mellonella* MNPV, *Aedes aegypti, Drosophila melanogaster, Spodoptera frugiperda*, and *Trichoplusia ni*. Thus, the

baculovirus transcriptional promoter can be, for example, a baculovirus immediate-early gene IEI or IEN promoter; an immediate-early gene in combination with a baculovirus delayed-early gene promoter region selected from the group consisting of a 39K and a *Hin*dIII fragment containing a delayed-early gene; or a baculovirus late gene promoter. The immediate-early or delayed-early promoters can be enhanced with transcriptional enhancer elements.

Particularly suitable for use herein is the strong polyhedrin promoter of the baculovirus, which directs a high level of expression of a DNA insert, as described in Friesen *et al.* (1986) "The Regulation of Baculovirus Gene Expression" in: THE MOLECULAR BIOLOGY OF BACULOVIRUSES (W.Doerfler, ed.); EP 127,839 and EP 155,476; and the promoter from the gene encoding the p10 protein, as described in Vlak *et al.*, *J. Gen. Virol.* (1988) *69*: 765-776. The plasmid for use herein usually also contains the polyhedrin polyadenylation signal, as described in Miller *et al.*, *Ann. Rev. Microbiol.* (1988) *42*: 177 and a procaryotic ampicillin-resistance (*amp*) gene and an origin of replication for selection and propagation in *E. coli*. DNA encoding suitable signal sequences can also be included and is generally derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene, as described in Carbonell *et al.*, *Gene* (1988) *73*: 409, as well as mammalian signal sequences such as those derived from genes encoding human α-interferon as described in Maeda *et al.*, *Nature* (1985) *315*: 592-594; human gastrin-releasing peptide, as described in Lebacq-Verheyden *et al.*, *Mol. Cell. Biol.* (1988) *8*: 3129; human IL-2, as described in Smith *et al.*, *Proc. Natl. Acad. Sci. USA* (1985) *82*: 8404; mouse IL-3, as described in Miyajima *et al.*, *Gene* (1987) *58*: 273; and human glucocerebrosidase, as described in Martin *et al.*, *DNA* (1988) 7:99.

Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified and can be used herein. See, for example, the description in Luckow et al., Bio/Technology(1988) *6:* 47-55, Miller *et al.*, in GENETIC ENGINEERING (Setlow, J.K. *et al.* eds.), Vol. 8 (Plenum Publishing, 1986), pp. 277-279, and Maeda *et al.*, *Nature,* (1985) *315*: 592-594. A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV. Such viruses may be used as the virus for transfection of host cells such as *Spodoptera frugiperda* cells.

Other baculovirus genes in addition to the polyhedrin promoter may be employed to advantage in a baculovirus expression system. These include immediate-early (alpha), delayed-early (beta), late (gamma), or very late (delta), according to the phase of the viral infection during which they are expressed. The expression of these genes occurs sequentially, probably as the result of a "cascade" mechanism of transcriptional regulation. Thus, the immediate-early genes are expressed immediately after infection, in the absence of other viral functions, and one or more of the resulting gene products induces transcription of the delayed-early genes. Some delayed-early gene products, in turn, induce transcription of late genes, and finally, the very late genes are expressed under the control of previously expressed gene products from one or more of the earlier classes. One relatively well defined component of this regulatory cascade is IEI, a preferred immediate-early gene of *Autographo californica* nuclear polyhedrosis virus (AcMNPV). IEI is pressed in the absence of other viral functions and encodes a product that stimulates the transcription of several genes of the delayed-early

class, including the preferred 39K gene, as described in Guarino and Summers, *J. Virol.* (1986) *57*: 563-571 and *J. Virol.* (1987) *61*: 2091-2099 as well as late genes, as described in Guanno and Summers, *Virol.* (1988) *162*: 444-451.

Immediate-early genes as described above can be used in combination with a baculovirus gene promoter region of the delayed-early category. Unlike the immediate-early genes, such delayed-early genes require the presence of other viral genes or gene products such as those of the immediate-early genes. The combination of immediate-early genes can be made with any of several delayed-early gene promoter regions such as 39K or one of the delayed-early gene promoters found on the *Hin*dIII fragment of the baculovirus genome. In the present instance, the 39 K promoter region can be linked to the foreign gene to be expressed such that expression can be further controlled by the presence of IEI, as described in L. A. Guarino and Summers (1986a), cited above; Guarino & Summers (1986b) *J. Virol.*, (1986) *60*: 215-223, and Guarino *et al.* (1986c), *J. Virol.* (1986) *60*: 224-229.

Additionally, when a combination of immediate-early genes with a delayed-early gene promoter region is used, enhancement of the expression of heterologous genes can be realized by the presence of an enhancer sequence in direct cis linkage with the delayed-early gene promoter region. Such enhancer sequences are characterized by their enhancement of delayed-early gene expression in situations where the immediate-early gene or its product is limited. For example, the hr5 enhancer sequence can be linked directly, in cis, to the delayed-early gene promoter region, 39K, thereby enhancing the expression of the cloned heterologous DNA as described in Guarino and Summers (1986a), (1986b), and Guarino *et al.* (1986).

The polyhedrin gene is classified as a very late gene. Therefore, transcription from the polyhedrin

promoter requires the previous expression of an unknown, but probably large number of other viral and cellular gene products. Because of this delayed expression of the polyhedrin promoter, state-of-the-art BEVs, such as the exemplary BEV system described by Smith and Summers in, for example, U.S. Pat. No., 4,745,051 will express foreign genes only as a result of gene expression from the rest of the viral genome, and only after the viral infection is well underway. This represents a limitation to the use of existing BEVs. The ability of the host cell to process newly synthesized proteins decreases as the baculovirus infection progresses. Thus, gene expression from the polyhedrin promoter occurs at a time when the host cell's ability to process newly synthesized proteins is potentially diminished for certain proteins such as human tissue plasminogen activator. As a consequence, the expression of secretory glycoproteins in BEV systems is complicated due to incomplete secretion of the cloned gene product, thereby trapping the cloned gene product within the cell in an incompletely processed form.

While it has been recognized that an insect signal sequence can be used to express a foreign protein that can be cleaved to produce a mature protein, the present invention is preferably practiced with a mammalian signal sequence for example the Factor VIII signal sequence.

An exemplary insect signal sequence suitable herein is the sequence encoding for a Lepidopteran adipokinetic hormone (AKH) peptide. The AKH family consists of short blocked neuropeptides that regulate energy substrate mobilization and metabolism in insects. In a preferred embodiment, a DNA sequence coding for a Lepidopteran *Manduca sexta* AKH signal peptide can be used. Other insect AKH signal peptides, such as those from the Orthoptera *Schistocerca gregaria* locus can also be employed to advantage. Another exemplary insect

signal sequence is the sequence coding for Drosophila cuticle proteins such as CPI, CP2, CP3 or CP4.

Currently, the most commonly used transfer vector that can be used herein for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, can also be used herein. Materials and methods for baculovirus/insect cell expression systems are commercially available in a kit form from companies such as Invitrogen (San Diego CA) ("MaxBac" kit). The techniques utilized herein are generally known to those skilled in the art and are fully described in Summers and Smith, A MANUAL OF METHODS FOR BACULOVIRUS VECTORS AND INSECT CELL CULTURE PROCEDURES, Texas Agricultural Experiment Station Bulletin No. 1555, Texas A&M University (1987); Smith *et al., Mol. Cell. Biol.* (1983) *3*: 2156, and Luckow and Summers (1989). These include, for example, the use of pVL985 which alters the polyhedrin start codon from ATG to ATT, and which introduces a *Bam*HI cloning site 32 basepairs downstream from the ATT, as described in Luckow and Summers, *Virology* (1989) *17*:31.

Thus, for example, for insect cell expression of the present polypeptides, the desired DNA sequence can be inserted into the transfer vector, using known techniques. An insect cell host can be cotransformed with the transfer vector containing the inserted desired DNA together with the genomic DNA of wild type baculovirus, usually by cotransfection. The vector and viral genome are allowed to recombine resulting in a recombinant virus that can be easily identified and purified. The packaged recombinant virus can be used to infect insect host cells to express a Factor VIII:C polypeptide analog.

Other methods that are applicable herein are the standard methods of insect cell culture, cotransfection and preparation of plasmids are set forth in Summers and Smith (1987), cited above. This reference

also pertains to the standard methods of cloning genes into AcMNPV transfer vectors, plasmid DNA isolation, transferring genes into the AcmMNPV genome, viral DNA purification, radiolabeling recombinant proteins and preparation of insect cell culture media. The procedure for the cultivation of viruses and cells are described in Volkman and Summers, *J. Virol.* (1975) *19*:820-832 and Volkman, *et al., J. Virol.* (1976) *19*:820-832.

Expression in Mammalian Cells

Mammalian expression systems can also be used to produce the Factor VIII:C polypeptide analogs and nucleic acid sequences encoding the analogs. Typical promoters for mammalian cell expression include the SV40 early promoter, the CMV promoter, the mouse mammary tumor virus LTR promoter, the adenovirus major late promoter (Ad MLP), and the herpes simplex virus promoter, among others. Other non-viral promoters, such as a promoter derived from the murine metallothionein gene, will also find use in mammalian constructs. Mammalian expression may be either constitutive or regulated (inducible), depending on the promoter. Typically, transcription termination and polyadenylation sequences will also be present, located 3' to the translation stop codon. Preferably, a sequence for optimization of initiation of translation, located 5' to the Factor VIII:C polypeptide analog coding sequence, is also present. Examples of transcription terminator/polyadenylation signals include those derived from SV40, as described in Sambrook et al. (1989) MOLECULAR CLONING: A LABORATORY MANUAL, 2d edition, (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.). Introns, containing splice donor and acceptor sites, may also be designed into the constructs of the present invention.

Enhancer elements can also be used herein to increase expression levels of the mammalian constructs. Examples include the SV40 early gene enhancer, as

described in Dijkema *et al.*, *EMBO J.* (1985) *4*: 761 and the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, as described in Gorman *et al.*, *Proc. Natl. Acad. Sci. USA* (1982b) *79*: 6777 and human cytomegalovirus, as described in Boshart *et al.*, *Cell* (1985) *41*: 521. A leader sequence can also be present which includes a sequence encoding a signal peptide, to provide for the secretion of the foreign protein in mammalian cells. Alternatively, the Factor VIII signal peptide can be used. Preferably, there are processing sites encoded between the leader fragment and the gene of interest such that the leader sequence can be cleaved either *in vivo* or *in vitro*. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

There exist expression vectors that provide for the transient expression in mammalian cells of DNA encoding the Factor VIII:C analog polypeptides. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful for purposes of identifying additional polypeptides that have Factor VIII:C-like activity.

Once complete, the mammalian expression vectors can be used to transform any of several mammalian cells. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include

dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216. A synthetic lipid particularly useful for polynucleotide transfection is N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride, which is commercially available under the name Lipofectin® (available from BRL, Gaithersburg, MD), and is described by Felgner *et al., Proc. Natl. Acad. Sci. USA* (1987) *84*:7413.

Mammalian cell lines available as hosts for expression are also known and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), human embryonic kidney cells, baby hamster kidney cells, mouse sertoli cells, canine kidney cells, buffalo rat liver cells, human lung cells, human liver cells, mouse mammary tumor cells, as well as others. The mammalian host cells used to produce the target polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.* (1979) *58*: 44, Barnes and Sato, *Anal. Biochem.* (1980) *102*: 255, U.S. Patent Nos. 4,767,704, 4,657,866, 4,927,762, or 4,560,655, WO 90/103430, WO 87/00195, and U.S. RE 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors such

as insulin, transferrin, or epidermal growth factor, salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin(tm) M drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The active Factor VIII:C analogs produced according to the invention have a variety of uses. For example, the analogs can be used as immunogens for the production of antibodies. The analogs can also be used for the treatment of hemophiliacs and other hosts having blood clotting disorders. In this regard, due to their resistance to proteolytic cleavage, the Factor VIII:C analogs may display increased plasma half-life or specific activity. Thus, the analogs may allow for lower dosages or alternative modes of administration and may improve hemostasis in hemophiliacs.

Alternatively, nucleic acid molecules or vectors comprising polynucleotide sequences encoding the Factor VIII:C analogs can be used directly for gene therapy and administered using standard gene delivery protocols. In this regard, the nucleotide sequences encoding the Factor VIII:C analogs can be stably integrated into the host cell genome or maintained on a stable episomal element in the host cell. Methods for gene delivery are known in the art. See, e.g., U.S. Patent No. 5,399,346.

A number of viral based systems have been developed for gene transfer into mammalian cells. For

example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems have been described (U.S. Patent No. 5,219,740; Miller and Rosman, *BioTechniques* (1989) 7:980-990; Miller, A.D., *Human Gene Therapy* (1990) *1*:5-14; Scarpa *et al.*, *Virology* (1991) *180*:849-852; Burns *et al.*, *Proc. Natl. Acad. Sci. USA* (1993) *90*:8033-8037; and Boris-Lawrie and Temin, *Cur. Opin. Genet. Develop.* (1993) *3*:102-109. A number of adenovirus vectors have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham, *J. Virol.* (1986) *57*:267-274; Bett *et al.*, *J. Virol.* (1993) *67*:5911-5921; Mittereder *et al.*, *Human Gene Therapy* (1994) *5*:717-729; Seth *et al.*, *J. Virol.* (1994) *68*:933-940; Barr *et al.*, *Gene Therapy* (1994) *1*:51-58; Berkner, K.L. *BioTechniques* (1988) *6*:616-629; and Rich *et al.*, *Human Gene Therapy* (1993) *4*:461-476).

Additionally, various adeno-associated virus (AAV) vector systems have been developed for gene delivery. Such systems can include control sequences, such as promoter and polyadenylation sites, as well as selectable markers or reporter genes, enhancer sequences, and other control elements which allow for the induction of transcription. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Patent Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 (published 23 January 1992) and WO 93/03769 (published 4 March 1993); Lebkowski *et al.*, *Molec. Cell. Biol.* (1988) *8*:3988-3996; Vincent *et al.*, *Vaccines 90* (1990) (Cold Spring Harbor Laboratory Press); Carter, B.J. *Current Opinion in Biotechnology*

(1992) *3*:533-539; Muzyczka, N. *Current Topics in Microbiol. and Immunol.* (1992) *158*:97-129; Kotin, R.M. *Human Gene Therapy* (1994) *5*:793-801; Shelling and Smith, *Gene Therapy* (1994) *1*:165-169; and Zhou *et al.*, *J. Exp. Med.* (1994) *179*:1867-1875.

Additional viral vectors which will find use for delivering the nucleic acid molecules encoding the Factor VIII:C analog polypeptides for gene transfer include those derived from the pox family of viruses, including vaccinia virus and avian poxvirus. By way of example, vaccinia virus recombinants expressing the novel Factor VIII:C analogs can be constructed as follows. The DNA encoding the particular analog is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant protein into the viral genome. The resulting TK^- recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

A vaccinia based infection/transfection system can be conveniently used to provide for inducible, transient expression of the Factor VIII:C analogs in a host cell. In this system, cells are first infected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into protein by the host

translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, e.g., Elroy-Stein and Moss, *Proc. Natl. Acad. Sci. USA* (1990) *87*:6743-6747; Fuerst *et al.*, *Proc. Natl. Acad. Sci. USA* (1986) *83*:8122-8126.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the Factor VIII:C analog genes. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an avipox vector is particularly desirable in human and other mammalian species since members of the avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, e.g., WO 91/12882; WO 89/03429; and WO 92/03545.

Molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael *et al.*, *J. Biol. Chem.* (1993) *268*:6866-6869 and Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) *89*:6099-6103, can also be used for gene delivery.

As an alternative approach to infection with vaccinia or avipox virus recombinants, or to the delivery of genes using other viral vectors, an amplification system can be used that will lead to high level expression following introduction into host cells. Specifically, a T7 RNA polymerase promoter preceding the coding region for T7 RNA polymerase can be engineered. Translation of RNA derived from this template will generate T7 RNA polymerase which in turn will transcribe more template. Concomitantly, there will be a cDNA whose expression is under the control of the T7 promoter. Thus, some of the T7 RNA polymerase generated from

translation of the amplification template RNA will lead to transcription of the desired gene. Because some T7 RNA polymerase is required to initiate the amplification, T7 RNA polymerase can be introduced into cells along with the template(s) to prime the transcription reaction.

The amplification template can be generated by PCR techniques. However the use of a plasmid is preferred. Since high level expression of T7 RNA polymerase appears to be lethal to host cells, the plasmid should be one where expression of T7 RNA polymerase can be controlled. For example, a lac operator can be engineered distal or proximal (or both) to the T7 promoter. The binding of the preexisting lac repressor in the appropriate bacterial strain would interfere with the transcription of the template by blocking access to the promoter by T7 RNA polymerase. Alternatively, or in combination with the above, a plasmid can be constructed where transcription from a bacterial promoter begins 3′ of the T7 gene and continues through the 5′ end of the T7 promoter. Such transcription will generate an antisense transcript and reduce or eliminate translation of T7 RNA polymerase RNAs. The second transcription unit consisting of the T7 promoter preceding the gene of interest can be provided by a separate plasmid or can be engineered onto the amplification plasmid. Colocalization of the two transcription units is beneficial for ease of manufacturing and ensures that both transcription units will always be together in the cells into which the plasmid is introduced. The T7 RNA polymerase plasmids may include UTRs which comprise an Internal Ribosome Entry Site (IRES) present in the leader sequences of picornaviruses such as the encephalomyocarditis virus (EMCV) UTR (Jang et al. *J. Virol.* (1989) *63*:1651-1660). This sequence serves to enhance expression of sequences under the control of the T7 promoter. For a further discussion of T7 systems and their use for transforming

cells, see, e.g., International Publication No. WO 94/26911; Studier and Moffatt, *J. Mol. Biol.* (1986) *189*:113-130; Deng and Wolff, *Gene* (1994) *143*:245-249; Gao *et al.*, *Biochem. Biophys. Res. Commun.* (1994) *200*:1201-1206; Gao and Huang, *Nuc. Acids Res.* (1993) *21*:2867-2872; Chen *et al.*, *Nuc. Acids Res.* (1994) *22*:2114-2120; and U.S. Patent No. 5,135,855.

Vectors encoding the subject Factor VIII:C analogs can also be packaged in liposomes prior to delivery to the subject or to cells derived therefrom. Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. The ratio of condensed DNA to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) *1097*:1-17; Straubinger *et al.*, in METHODS OF ENZYMOLOGY (1983), Vol. 101, pp. 512-527.

Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations, with cationic liposomes particularly preferred. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) *84*:7413-7416); mRNA (Malone *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) *86*:6077-6081); and purified transcription factors (Debs *et al.*, *J. Biol. Chem.* (1990) *265*:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl]-N,N,N-triethyl-ammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) *84*:7413-7416). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boerhinger). Other cationic liposomes can be prepared

from readily available materials using techniques well known in the art. See, e.g., Szoka *et al.*, *Proc. Natl. Acad. Sci. USA* (1978) *75*:4194-4198; PCT Publication No. WO 90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphoshatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilammelar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger *et al.*, in METHODS OF IMMUNOLOGY (1983), Vol. 101, pp. 512-527; Szoka *et al.*, *Proc. Natl. Acad. Sci. USA* (1978) *75*:4194-4198; Papahadjopoulos *et al.*, *Biochim. Biophys. Acta* (1975) *394*:483; Wilson *et al.*, *Cell* (1979) *17*:77); Deamer and Bangham, *Biochim. Biophys. Acta* (1976) *443*:629; Ostro *et al.*, *Biochem. Biophys. Res. Commun.* (1977) *76*:836; Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* (1979) *76*:3348); Enoch and Strittmatter, *Proc. Natl. Acad. Sci. USA* (1979) *76*:145); Fraley *et al.*, *J. Biol. Chem.* (1980) *255*:10431; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* (1978) *75*:145; and Schaefer-Ridder *et al.*, *Science* (1982) *215*:166.

The recombinant vectors (whether or not encapsulated in liposomes), may be administered in pharmaceutical compositions as described above. The

pharmaceutical compositions will comprise sufficient genetic material to produce a therapeutically effective amount of the analog or analogs, as described above. For purposes of the present invention, an effective dose will be from about 0.05 mg/kg to about 50 mg/kg of the DNA constructs in the individual to which it is administered. Once formulated, the compositions of the invention can be administered directly to the subject or, alternatively, in the case of the vectors described above, delivered *ex vivo*, to cells derived from the subject. Methods for the *ex vivo* delivery and reimplantation of transformed cells into a subject are known in the art and described in e.g., International Publication No. WO 93/14778 (published 5 August 1993). Generally, such methods will include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei, all well known in the art.

Direct delivery of the compositions will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Although any similar or equivalent methods and materials may be employed in the practice or testing of the present invention, the preferred methods and materials are now described.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

Examples

The Factor VIII:C polypeptide analogs are made using conventional mutagenesis techniques. In this regard, mutagenesis of the Factor VIII:C nucleotide sequences can be performed utilizing plasmids which include sequences encoding the full-length molecule, plasmids encoding the light and heavy chains and various modifications of these molecules, depending on the Factor VIII:C analog desired. Such plasmids are known and described in, e.g., U.S. Patent no. 5,045,455. The plasmids are first linearized e.g., by using restriction endonucleases which cleave at unique restriction sites. Once linearized, the plasmids are treated with calf intestine phosphatase and separated on low melting temperature tris-acetate agarose gels. The linearized band is extracted by adsorption to silica dioxide and eluted in tris-EDTA. The plasmid is then denatured and the desired phosphorylated mutagenic oligonucleotide is added. The mixture is heated and allowed to slowly cool at room temperature. A heteroduplex oligonucleotide mixture can be used and the reactions made with, e.g., 2 mM $MgCl_2$, 1mM beta-mercaptoethanol, 400 μM ATP, 100 μM deoxynucleotide triphosphate, 3-4 units/μL of Klenow fragment of *E. coli* DNA polymerase I and 400 units/μL of T4 DNA ligase.

The reactions are terminated using phenolchloroform extraction and ethanol precipitation. DNA obtained is used to transform bacterial host cells and positive clones selected. DNA from the clones is transferred to nitrocellulose, filters prepared, and hybridized to screening probes to ensure that the mutagenic oligonucleotide is introduced into the correct fragment. Final mutations are confirmed by DNA sequencing.

The DNA can be prepared by banding in CsCl and can be used to transfect COS-1 monkey cells as described in Kaufman, *PNAS* (1982) *82*:689. After transfection, the

polypeptide analog is isolated and Factor VIII:C activity is assayed by the Kabi Coatest chromagenic assay method for the ability to clot Factor VIII deficient plasma before and after thrombin activation.

Example 1

Construction of a Factor VIII:C Polypeptide Analog Pro221

Residue 221 of Factor VIII:C is mutated using the oligonucleotide TTC ATG CAG GAT AGG CCX GCT GCA TCT GCT CGG. The GAT encoding for Asp which normally occurs at the underlined position is mutated to form the codon for Pro which can be CCX where X is A, T, G or C. The mutagenesis is carried out, the correct sequence is confirmed, and the analog produced and tested for activity, as described above.

Other Factor VIII:C polypeptide analogs can be constructed and assayed as described above.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An active Factor VIII:C polypeptide analog comprising a native Factor VIII:C polypeptide that is modified at a site adjacent to a non-activating Arg residue, wherein the modification comprises creation of an Arg-Pro or a Pro-Arg dipeptide.

2. The analog of claim 1, wherein the non-activating Arg residue is an Arg residue selected from the group consisting of amino acid residues 220, 226, 250, 279, 282, 336, 359, 562, 747, 776, 1310, 1313, 1645, 1648, 1719, and 1721, numbered with respect to the native Factor VIII:C polypeptide sequence.

3. The analog of claim 1, wherein the modification is an amino acid addition, substitution, or deletion, or a combination thereof.

4. The analog of claim 1, wherein the modification is amino acid substitution and the substitution is of about one to about 7 amino acid residues.

5. The analog of claim 1, wherein the modification is amino acid deletion, and the deletion is of about one to about 7 amino acid residues.

6. An active Factor VIII:C polypeptide analog comprising a native Factor VIII:C polypeptide that is modified at a site adjacent to a non-activating Arg residue, wherein the modification comprises creation of a tripeptide having the formula P_3-P_2-P_1, wherein P_3 is a residue selected from the group consisting of Phe, Glu, and Pro; P_2 is any amino acid residue except Ser and P2 is not Leu335 and is not Asn1720; and P_1 is Arg.

7. The analog of claim 2, wherein the non-activating Arg residue is Arg220, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Asp219 and Arg 220; (b) an insertion of at least one Pro residue between Arg220 and Asp221; and (c) a deletion comprising residues Asp221, Ala222, Ala223, Ser224, Ala225, Arg226, Ala227, and Trp228.

8. The analog of claim 6, wherein the non-activating Arg residue is Arg220, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Gln218 and Asp219; (b) an insertion of at least one Glu residue between Gln218 and Asp219; (c) an insertion of at least one Pro residue between Gln218 and Asp219; (d) a deletion comprising residues Thr212, Lys213, Asn214, Ser215, Leu216, Met217, and Gln218; (e) a substitution of at least one Phe residue at Gln218; (f) a substitution of at least one Glu residue at Gln218; and (g) a substitution of at least one Pro residue at Gln218.

9. The analog of claim 2, wherein the non-activating Arg residue is Arg226, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Ala225 and Arg226; (b) an insertion of at least one Pro residue between Arg226 and Ala227; (c) a deletion comprising residues Ala227 and Trp228; (d) a deletion comprising residues Ala227 and Trp228 and insertion of at least one Pro residue to replace the deleted residues; and (e) a deletion comprising residues Ala227, Trp228, Pro229, and Lys230, and an insertion of at least one Pro residue to replace the deleted residues.

10. The analog of claim 6, wherein the non-activating Arg residue is Arg226, and the modification

comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Ser224 and Ala225; (b) an insertion of at least one Glu residue between Ser224 and Ala225; (c) an insertion of at least one Pro residue between Ser224 and Ala225; (d) a substitution of at least one Phe residue at Ser224; (e) a substitution of at least one Glu residue at Ser224; and (f) a substitution of at least one Pro residue at Ser224.

11. The analog of claim 2, wherein the non-activating Arg residue is Arg250, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between His249 and Arg250; (b) an insertion of at least one Pro residue between Arg250 and Lys251; (c) a deletion comprising residues Lys251 and Ser252 and insertion of at least one Pro residue to replace the deleted residues; (d) a substitution of Lys251 with at least one Pro residue; (e) a deletion comprising residues Gly244, Leu245, Ile246, Gly247, Cys248, and His249; and (f) a deletion comprising residues Arg240, Ser241, Leu242, Pro243, Gly244, Leu245, Ile246, Gly247, Cys248, and His249 and an insertion of at least one Pro residue to replace the deleted residues.

12. The analog of claim 6, wherein the non-activating Arg residue is Arg250, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Cys248 and His249; (b) an insertion of at least one Glu residue between Cys248 and His249; (c) an insertion of at least one Pro residue between Cys248 and His249; (d) a deletion comprising residues Gly244, Leu245, Ile246, Gly247, and Cys248; (e) a substitution of at least one Phe residue at Cys248; (f) a substitution of at least one Glu residue at Cys248; and (g) a substitution of at least one Pro residue at Cys248.

13. The analog of claim 2, wherein the non-activating Arg residue is Arg279, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Val278 and Arg279; (b) an insertion of at least one Pro residue between Arg279 and Asn280; (c) a deletion comprising residues Asn280, His281, and Arg282 and an insertion of at least one Pro residue to replace the deleted residues; and (d) a deletion comprising Asn280, His281, Arg282, Gln283, Ala284, and Ser285, and an insertion of at least one Pro residue to replace the deleted residues.

14. The analog of claim 6, wherein the non-activating Arg residue is Arg279, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Leu277 and Val278; (b) an insertion of at least one Glu residue between Leu277 and Val278; (c) an insertion of at least one Pro residue between Leu277 and Val278; (d) a deletion comprising residue Leu277; (e) a deletion comprising residue Val278; (f) a deletion comprising residues Gly273, His274, Thr275, Phe276, and Leu277; (g) a deletion comprising residues Leu271, Glu272, Gly273, His274, Thr275, Phe276, and Leu277; (h) a substitution of at least one Phe residue at Leu277; (i) a substitution of at least one Glu residue at Leu277; and (j) a substitution of at least one Pro residue at Leu277.

15. The analog of claim 2, wherein the non-activating Arg residue is Arg282, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between His281 and Arg282; (b) an insertion of at least one Pro residue between Arg282 and Gln283; (c) a deletion comprising residues Arg279, Asn280, and His281 and an insertion of at least one Pro residue to replace the

deleted residues; (d) a deletion comprising residues Gln283, Ala284, and Ser285, and an insertion of at least one Pro residue to replace the deleted residues; and (e) a deletion comprising residues Gln283, Ala284, Ser285, Leu286, Glu287, Ile288, and Ser289.

16. The analog of claim 6, wherein the non-activating Arg residue is Arg282, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Asn280 and His281; (b) an insertion of at least one Glu residue between Asn280 and His281; (c) an insertion of at least one Pro residue between Asn280 and His281; (d) a deletion comprising residues Leu277, Val278, Arg279, and Asn280; (e) a deletion comprising residues Gly273, His274, Thr275, Phe276, Leu277, Val278, Arg279, and Asn280; (f) a substitution of at least one Phe residue at Asn280; (g) a substitution of at least one Glu residue at Asn280; and (h) a substitution of at least one Pro residue at Asn280.

17. The analog of claim 2, wherein the non-activating Arg residue is Arg336, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Leu335 and Arg336; (b) a deletion comprising residues Gln334, and Leu335; (c) an insertion of at least one Pro residue between Arg336 and Met337; and (d) a deletion comprising residues Met337, and Lys338 and insertion of at least one Pro residue in place of the deleted residues.

18. The analog of claim 6, wherein the non-activating Arg residue is Arg336, and the modification comprises at least one selected from the group consisting of: (a) a deletion comprising residue Leu335 and an insertion of at least one Phe residue between Pro333 and

Gln334; (b) a deletion comprising residue Leu335 and an insertion of at least one Glu residue between Pro333 and Gln334; (c) a deletion comprising residue Leu335 and an insertion of at least one Pro residue between Pro333 and Gln334; (d) a deletion comprising residue Leu335; (e) a deletion comprising residues Gln334, and Leu335; (f) a deletion comprising residues Pro333, Gln334, and Leu335; (g) a deletion comprising residues Glu332, Pro333, Gln334, and Leu335; (h) a deletion comprising residue Leu335 and a substitution of at least one Phe residue at Pro333; and (i) a deletion comprising residue Leu335 and a substitution of at least one Glu residue at Pro333.

19. The analog of claim 2, wherein the non-activating Arg residue is Arg359, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Val358 and Arg359; (b) an insertion of at least one Pro residue between Arg359 and Phe360; (c) a deletion comprising residues Phe360, Asp361, Asp362, Asp363, Asn364, and Ser365; and (d) a deletion comprising residues Phe360, Asp361, Asp362, Asp363, Asn364, Ser365, Pro366, and Ser367, and an insertion of at least one Pro residue to replace the deleted residues.

20. The analog of claim 6, wherein the non-activating Arg residue is Arg359, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Val357 and Val358; (b) an insertion of at least one Glu residue between Val357 and Val358; (c) an insertion of at least one Pro residue between Val357 and Val358; (d) a deletion comprising residues Met355, Asp356, and Val357; (e) a substitution of at least one Phe residue at Val357; (f) a substitution of at least one Glu residue at Val357; and (g) a substitution of at least one Pro residue at Val357.

21. The analog of claim 2, wherein the non-activating Arg residue is Arg562, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Gln561 and Arg562; (b) an insertion of at least one Pro residue between Arg562 and Gly563; (c) a deletion comprising residues Ser558, Val559, Asp560, Gln561, and an insertion of at least one Pro residue to replace the deleted residues; (d) a deletion comprising residues Lys556, Glu557, Ser558, Val559, Asp560, Gln561, and an insertion of at least one Pro residue to replace the deleted residues; (e) a deletion comprising residues Gly563, Asn564, Gln565, Ile566, Met567, and Ser568, and an insertion of at least one Pro residue to replace the deleted residues; and (f) a deletion comprising residues Gly563, Asn564, Gln565, Ile566, Met567, Ser568, Asp569, Lys570, and Arg571, and an insertion of at least one Pro residue to replace the deleted residues.

22. The analog of claim 6, wherein the non-activating Arg residue is Arg562, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Asp560 and Gln561; (b) an insertion of at least one Glu residue between Asp560 and Gln561; (c) an insertion of at least one Pro residue between Asp560 and Gln561; (d) a deletion comprising residues Ser558, Val559, and Asp560; (e) a substitution of at least one Phe residue at Asp560; (f) a substitution of at least one Glu residue at Asp560; and (g) a substitution of at least one Pro residue at Asp560.

23. The analog of claim 2, wherein the non-activating Arg residue is Arg747, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Ser746 and Arg747; (b) a deletion comprising residue

Ser746 and an insertion of at least one Pro residue to replace the deleted residue; (c) a deletion comprising residue His748; (d) a deletion comprising residue His748 and an insertion of at least one Pro residue to replace the deleted residue; (e) an insertion of at least one Pro residue between Arg747 and His748; (f) a deletion comprising residues His748, Pro749, and Ser750 and an insertion of at least one Pro residue to replace the deleted residues; (g) a deletion comprising Ser743, Gln744, Asn745, Ser746; and (h) a deletion comprising His748, Pro749, Ser750, Thr751, Arg752, Gln753, and Lys754, and an insertion of at least one Pro residue to replace the deleted residues.

24. The analog of claim 6, wherein the non-activating Arg residue is Arg747, and the modification comprises at least one selected from the group consisting of: (a) a deletion comprising residue Ser746 and an insertion of at least one Phe residue between Gln744 and Asn745; (b) a deletion comprising residue Ser746 and an insertion of at least one Glu residue between Gln744 and Asn745; (c) a deletion comprising residue Ser746 and an insertion of at least one Pro residue between Gln744 and Asn745; (d) a deletion comprising residue Ser746 and a substitution of at least one Phe residue at Gln744; (e) a deletion comprising residue Ser746 and a substitution of at least one Glu residue at Gln744; and (f) a deletion comprising residue Ser746 and a substitution of at least one Pro residue at Gln744.

25. The analog of claim 2, wherein the non-activating Arg residue is Arg776, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between His775 and Arg776; (b) an insertion of at least one Pro residue between Arg776 and Thr777; (c) a deletion comprising residue Thr777; (d) a deletion comprising

residue Thr777 and an insertion of at least one Pro residue to replace the deleted residue; (e) a deletion comprising residues Trp772, Phe773, Ala774, and His775; (f) a deletion comprising residues Trp772, Phe773, Ala774, and His775, and an insertion of at least one Pro residue to replace the deleted residues; (g) a deletion comprising residues Lys768, Thr769, Asp770, Pro771, Trp772, Phe773, Ala774, and His775, and an insertion of at least one Pro residue to replace the deleted residues; and (h) a deletion comprising residues Thr777, Pro778, met779, Pro780, and Lys781, and an insertion of at least one Pro residue to replace the deleted residues.

26. The analog of claim 6, wherein the non-activating Arg residue is Arg776, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Ala774 and His775; (b) an insertion of at least one Glu residue between Ala774 and His775; (c) an insertion of at least one Pro residue between Ala774 and His775; (d) a deletion comprising residue Ala774; (e) a deletion comprising residues Trp772, Phe773 and Ala774; (f) a deletion comprising residues Lys768, Thr769, Asp770, Pro771, Trp772, and Phe773; (g) a substitution of at least one Phe residue at Ala774; (h) a substitution of at least one Glu residue at Ala774; and (i) a substitution of at least one Pro residue at Ala774.

27. The analog of claim 2, wherein the non-activating Arg residue is Arg1310, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Gln1309 and Arg1310; (b) an insertion of at least one Pro residue between Arg1310 and Ser1311; (c) a deletion comprising residue Ser1311 and an insertion of at least one Pro residue to replace the residue; (d) a deletion comprising residues Ser1311, Lys1312, and Arg1313 and an

insertion of at least one Pro residue to replace the deleted residues; (e) a deletion comprising residues Ser1311, Lys1312, Arg1313, Ala1314, Leu1315, and Lys1316, and an insertion of at least one Pro residue to replace the deleted residues; (f) a deletion comprising residues Ser1311, Lys1312, Arg1313, Ala1314, Leu1315, Lys1316, Gln1317, Phe1318, Arg1319, and Leu1320; and (g) a deletion comprising residues Ser1311, Lys1312, Arg1313, Ala1314, Leu1315, Lys1316, Gln1317, Phe1318, Arg1319, and Leu1320, and an insertion of at least one Pro residue to replace the deleted residues.

28. The analog of claim 6, wherein the non-activating Arg residue is Arg1310, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Thr1308 and Gln1309; (b) an insertion of at least one Glu residue between Thr1308 and Gln1309; (c) an insertion of at least one Pro residue between Thr1308 and Gln1309; (d) a deletion comprising residues Val1307, and Thr1308; (e) a substitution of at least one Phe residue at Thr1308; (f) a substitution of at least one Glu residue at Thr1308; and (g) a substitution of at least one Pro residue at Thr1308.

29. The analog of claim 2, wherein the non-activating Arg residue is Arg1313, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Pro residue between Lys1312 and Arg1313; (b) a deletion comprising residue Lys1312 and an insertion of at least one Pro residue to replace the deleted residue; (c) a deletion comprising residues Arg1310, Ser1311, and Lys1312, and an insertion of at least one Pro residue to replace the deleted residues; (d) an insertion of at least one Pro residue between Arg1313 and Ala1314; (e) a deletion comprising residues Ala1314, Leu1315, and Lys1316, and an insertion

of at least one Pro residue to replace the deleted residues; (f) a deletion comprising residues Ala1314, Leu1315, Lys1316, Gln1317, Phe1318, and Arg1319 and an insertion of at least one Pro residue to replace the deleted residues; and (g) a deletion comprising residues Ala1314, Leu1315, Lys1316, Gln1317, Phe1318, Arg1319, and Leu1320.

30. The analog of claim 6, wherein the non-activating Arg residue is Arg1313, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Ser1311 and Lys1312; (b) an insertion of at least one Glu residue between Ser1311 and Lys1312; (c) an insertion of at least one Pro residue between Ser1311 and Lys1312; (d) a deletion comprising residues Val1307, Thr1308, Gln1309, Arg1310, and Ser1311; (e) a substitution of at least one Phe residue at Ser1311; (f) a substitution of at least one Glu residue at Ser 1311; and (g) a substitution of at least one Pro residue at Ser 1311.

31. The analog of claim 2, wherein the non-activating Arg residue is Arg1645, and the modification comprises at least one selected from the group consisting of: (a) a deletion comprising residues Val1642, Leu1643, and Lys1644; (b) a deleteion comprising residue Lys1644 and an insertion of at least one Pro residue to replace the deleted residue; (c) an insertion of at least one Pro residue between Arg1645 and Lys1644; (d) an insertion of at least one Pro residue between Arg1645 and His1646; (e) a deletion comprising residues His1646, Gln1647, and Arg1648 and an insertion of at least one Pro residue to replace the deleted residues; and (f) a deletion comprising residues His1646, Gln1647, Arg1648, Glu1649, Ile1650, Thr1651, and Arg1652, and an insertion of at least one Pro residue to replace the deleted residues.

32. The analog of claim 6, wherein the non-activating Arg residue is Arg1645, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Leu1643 and Lys1644; (b) an insertion of at least one Glu residue between Leu1643 and Lys1644; (c) an insertion of at least one Pro residue between Leu1643 and Lys1644; (d) a deletion comprising residues Val1642, and Leu1643; (e) a deletion comprising residues Pro1641, Val1642, and Leu1643; (f) a substitution of at least one Phe residue at Leu1643; (g) a substitution of at least one Glu residue at Leu1643; and (h) a substitution of at least one Pro residue at Leu1643.

33. The analog of claim 2, wherein the non-activating Arg residue is Arg1648, and the modification comprises at least one selected from the group consisting of: (a) a deletion comprising residues Val1642, Leu1643, Lys1644, Arg1645, His1646, and Gln1647; (b) an insertion of at least one Pro residue between Gln1647 and Arg1648; (c) a deletion comprising residues Lys1644, Arg1645, His1646, and Gln1647 and an insertion of at least one Pro residue to replace the deleted residues; (d) a deletion comprising residues Glu1649, Ile1650, Thr1651, and Arg1652 and an insertion of at least one Pro residue to replace the deleted residues; and (e) an insertion of at least one Pro residue between Arg1648 and Glu1649.

34. The analog of claim 6, wherein the non-activating Arg residue is Arg1648, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between His1646 and Gln1647; (b) an insertion of at least one Glu residue between His1646 and Gln1647; (c) an insertion of at least one Pro residue between His1646 and Gln1647; (d) a deletion comprising residues Val1642, Leu1643, Lys1644, Arg1645, and His1646; (e) a deletion comprising residues

Pro1641, Val1642, Leu1643, Lys1644, Arg1645, and His1646; (f) a substitution of at least one Phe residue at His1646; (g) a substitution of at least one Glu residue at His1646; and (h) a substitution of at least one Pro residue at His1646.

35. The analog of claim 2, wherein the non-activating Arg residue is Arg1719, and the modification comprises at least one selected from the group consisting of: (a) a deletion comprising residues His1716, Val1717, and Leu1718; (b) an insertion of at least one Pro residue between Leu1718 and Arg1719; (c) a deletion comprising residues Asn1720, and Arg1721 and an insertion of at least one Pro residue to replace the deleted residues; and (d) an insertion of at least one Pro residue between Arg1719 and Asn1720.

36. The analog of claim 6, wherein the non-activating Arg residue is Arg1719, and the modification comprises at least one selected from the group consisting of: (a) an insertion of at least one Phe residue between Val1717 and Leu1718; (b) an insertion of at least one Glu residue between Val1717 and Leu1718; (c) an insertion of at least one Pro residue between Val1717 and Leu1718; (d) a deletion comprising residues His1716, and Val1717; (e) a substitution of at least one Phe residue at Val1717; and (f) a substitution of at least one Glu residue at Val1717; (g) a substitution of at least one Pro residue at Val1717.

37. The analog of claim 2, wherein the non-activating Arg residue is Arg1721, and the modification comprises at least one selected from the group consisting of: (a) a deletion comprising residues His1716, Val1717, Leu1718, Arg1719, and Asn1720; (b) an insertion of at least one Pro residue between Asn1720 and Arg1721; (c) an insertion of at least one Pro residue between Arg1721 and

Ala1722; (d) a deletion comprising residues Ala1722, Gln1723, and Ser1724 and an insertion of at least one Pro residue to replace the deleted residues; (e) a deletion comprising residues Ala1722, Gln1723, Ser1724, Gly1725, Ser1726, and Val1727; and (f) a deletion comprising residues Ala1722, Gln1723, Ser1724, Gly1725, and Ser1726, and an insertion of at least one Pro residue to replace the deleted residues.

38. The analog of claim 6, wherein the non-activating Arg residue is Arg1721, and the modification comprises at least one selected from the group consisting of: (a) a deletion comprising residue Asn1720 and an insertion of at least one Phe residue between Leu1718 and Arg1719; (b) a deletion comprising residue Asn1720 and an insertion of at least one Glu residue between Leu1718 and Arg1719; (c) a deletion comprising residue Asn1720 and an insertion of at least one Pro residue between Leu1718 and Arg1719; (d) a deletion comprising residues Val1717, Leu1718, Arg1719, and Asn1720; (e) a deletion comprising residue Asn1720 and a substitution of at least one Phe residue at Leu1718; (f) a deletion comprising residue Asn1720 and a substitution of at least one Glu residue at Leu1718; and (g) a deletion comprising residue Asn1720 and a substitution of at least one Pro residue at Leu1718;

39. An active Factor VIII:C polypeptide analog comprising a native Factor VIII:C polypeptide that is modified at at least one non-activating Arg residue selected from the group consisting of Arg336, Arg1719 and Arg1721, wherein the modification comprises a substitution of any of amino acids Pro, Glu, Asp, Asn, Gln, Ser and Tyr for Arg336, a substitution of any of amino acids Pro, Glu, Asp, Asn, Gln, Ser and Tyr for Arg1719 and/or a substitution of any of amino acids Glu,

Asp, Asn, Gln, Ser and Tyr for Arg1721, numbered with respect to the native Factor VIII:C polypeptide sequence.

40. The analog of claim 39, wherein the modification comprises at least one amino acid substitution selected from the group consisting of a substitution of Pro for Arg336, a substitution of Pro for Arg1719 and a substitution of Glu for Arg1721, numbered with respect to the native Factor VIII:C polypeptide sequence.

41. The analog of claim 1, wherein the native Factor VIII:C polypeptide is selected from the group consisting of:

a) a full-length Factor VIII:C molecule comprising a signal peptide and all A, B, and C domains;

b) a mature Factor VIII:C molecule comprising all A, B, and C domains and lacking a signal peptide;

c) a truncated Factor VIII:C molecule lacking a signal peptide and at least a portion of the B domain;

d) a cleaved Factor VIII:C molecule comprising a light chain subunit of molecular weight of about 80 kD;

e) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight in a range of about 90 kD to 200 kD;

f) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 90 kD;

g) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 50 kD;

h) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 43 kD; and

i) a cleaved Factor VIII:C molecule comprising a light chain fragment of molecular weight of about 73 kD.

42. An active Factor VIII:C polypeptide analog complex comprising at least two Factor VIII:C polypeptide analogs as claimed in claim 41, or at least one Factor VIII:C polypeptide analog and at least one Factor VIII:C polypeptide, wherein the complex comprises a metal ion.

43. The analog complex of claim 41, wherein the complex comprises two Factor VIII:C polypeptide analogs, and the Factor VIII:C polypeptides that are modified to form the two analogs are selected from the group consisting of molecular weights of about (a) 80 kD and 90 kD; (b) 73 kD and 90 kD; (c) 80 kD and 50 kD; (d) 80 kD and 43 kD; (e) 73 kD and 50 kD; and (f) 73 kD and 43 kD.

44. The analog complex of claim 42, wherein the metal ion is a divalent cation.

45. The analog complex of claim 44, wherein the divalent cation is a Ca^{++} ion or a Cu^{++} ion.

46. The analog of claim 6, wherein the native Factor VIII:C polypeptide is selected from the group consisting of:

a) a full-length Factor VIII:C molecule comprising a signal peptide and all A, B, and C domains;

b) a mature Factor VIII:C molecule comprising all A, B, and C domains and lacking a signal peptide;

c) a truncated Factor VIII:C molecule lacking a signal peptide and at least a portion of the B domain;

d) a cleaved Factor VIII:C molecule comprising a light chain subunit of molecular weight of about 80 kD;

e) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight in a range of about 90 kD to 200 kD;

f) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 90 kD;

g) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 50 kD;

h) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 43 kD; and

i) a cleaved Factor VIII:C molecule comprising a light chain fragment of molecular weight of about 73 kD.

47. An active Factor VIII:C polypeptide analog complex comprising at least two Factor VIII:C polypeptide analogs as claimed in claim 46, or at least one Factor VIII:C polypeptide analog and at least one Factor VIII:C polypeptide, wherein the complex comprises a metal ion.

48. The analog complex of claim 46, wherein the complex comprises two Factor VIII:C polypeptide analogs, and the Factor VIII:C polypeptides that are modified to form the two analogs are selected from the group consisting of molecular weights of about (a) 80 kD and 90 kD; (b) 73 kD and 90 kD; (c) 80 kD and 50 kD; (d) 80 kD and 43 kD; (e) 73 kD and 50 kD; and (f) 73 kD and 43 kD.

49. The analog complex of claim 47, wherein the metal ion is a divalent cation.

50. The analog complex of claim 49, wherein the divalent cation is a Ca^{++} ion or a Cu^{++} ion.

51. The analog of claim 40, wherein the native Factor VIII:C polypeptide is selected from the group consisting of:

a) a full-length Factor VIII:C molecule comprising a signal peptide and all A, B, and C domains;

b) a mature Factor VIII:C molecule comprising all A, B, and C domains and lacking a signal peptide;

c) a truncated Factor VIII:C molecule lacking a signal peptide and at least a portion of the B domain;

d) a cleaved Factor VIII:C molecule comprising a light chain subunit of molecular weight of about 80 kD;

e) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight in a range of about 90 kD to 200 kD;

f) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 90 kD;

g) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 50 kD;

h) a cleaved Factor VIII:C molecule comprising a heavy chain fragment of molecular weight of about 43 kD; and

i) a cleaved Factor VIII:C molecule comprising a light chain fragment of molecular weight of about 73 kD.

52. An active Factor VIII:C polypeptide analog complex comprising at least two Factor VIII:C polypeptide analogs as claimed in claim 51, or at least one Factor

VIII:C polypeptide analog and at least one Factor VIII:C polypeptide, wherein the complex comprises a metal ion.

53. The analog complex of claim 51, wherein the complex comprises two Factor VIII:C polypeptide analogs, and the Factor VIII:C polypeptides that are modified to form the two analogs are selected from the group consisting of molecular weights of about (a) 80 kD and 90 kD; (b) 73 kD and 90 kD; (c) 80 kD and 50 kD; (d) 80 kD and 43 kD; (e) 73 kD and 50 kD; and (f) 73 kD and 43 kD.

54. The analog complex of claim 52, wherein the metal ion is a divalent cation.

55. The analog complex of claim 54, wherein the divalent cation is a Ca^{++} ion or a Cu^{++} ion.

56. A method of producing a Factor VIII:C polypeptide analog, comprising:

a) providing a native Factor VIII:C polypeptide that comprises an amino acid sequence; and

b) modifying an amino acid residue in the amino acid sequence to produce the analog of claim 1.

57. A method of producing a Factor VIII:C polypeptide analog, comprising:

a) providing a native Factor VIII:C polypeptide that comprises an amino acid sequence; and

b) modifying an amino acid residue in the amino acid sequence to produce the analog of claim 6.

58. A method of producing a Factor VIII:C polypeptide analog, comprising:

a) providing a native Factor VIII:C polypeptide that comprises an amino acid sequence; and

b) modifying an amino acid residue in the amino acid sequence to produce the analog of claim 40.

59. A nucleic acid molecule comprising a nucleotide sequence that encodes the Factor VIII:C polypeptide analog of claim 1.

60. A nucleic acid molecule comprising a nucleotide sequence that encodes the Factor VIII:C polypeptide analog of claim 6.

61. A nucleic acid molecule comprising a nucleotide sequence that encodes the Factor VIII:C polypeptide analog of claim 40.

62. A recombinant vector comprising the nucleic acid molecule of claim 59 and a regulatory element, wherein the nucleic acid molecule is under regulatory control of the regulatory element.

63. A recombinant vector comprising the nucleic acid molecule of claim 60 and a regulatory element, wherein the nucleic acid molecule is under regulatory control of the regulatory element.

64. A recombinant vector comprising the nucleic acid molecule of claim 61 and a regulatory element, wherein the nucleic acid molecule is under regulatory control of the regulatory element.

65. A recombinant host cell comprising the nucleic acid molecule of claim 59.

66. A recombinant host cell comprising the nucleic acid molecule of claim 60.

67. A recombinant host cell comprising the nucleic acid molecule of claim 61.

68. A recombinant host cell comprising the recombinant vector of claim 62.

69. A recombinant host cell comprising the recombinant vector of claim 63.

70. A recombinant host cell comprising the recombinant vector of claim 64.

71. A method of producing an active Factor VIII:C polypeptide analog comprising:

a) providing the recombinant host cell of claim 65; and

b) allowing the recombinant host cell to express the analog.

72. A method of producing an active Factor VIII:C polypeptide analog comprising:

a) providing the recombinant host cell of claim 66; and

b) allowing the recombinant host cell to express the analog.

73. A method of producing an active Factor VIII:C polypeptide analog comprising:

a) providing the recombinant host cell of claim 67; and

b) allowing the recombinant host cell to express the analog.

74. A method of producing an active Factor VIII:C polypeptide analog comprising:

a) providing the recombinant host cell of claim 68; and

b) allowing the recombinant host cell to express the analog.

75. A method of producing an active Factor VIII:C polypeptide analog comprising:

a) providing the recombinant host cell of claim 69; and

b) allowing the recombinant host cell to express the analog.

76. A method of producing an active Factor VIII:C polypeptide analog comprising:

a) providing the recombinant host cell of claim 70; and

b) allowing the recombinant host cell to express the analog.

77. A method of producing a nucleic acid molecule that encodes a Factor VIII:C polypeptide analog comprising:

a) providing a nucleic acid molecule that encodes a native Factor VIII:C polypeptide, wherein the nucleic acid molecule comprises a codon for each amino acid residue in the native Factor VIII:C polypeptide; and

b) modifying at least one codon that is adjacent to a codon encoding a non-activating Arg residue, to produce the nucleic acid molecule of claim 59.

78. The method of producing a nucleic acid molecule as claimed in claim 77, wherein the modifying is performed by site directed mutagenesis or by use of polymerase chain reaction techniques.

79. A method of producing a nucleic acid molecule that encodes a Factor VIII:C polypeptide analog comprising:

a) providing a nucleic acid molecule that encodes a native Factor VIII:C polypeptide, wherein the nucleic acid molecule comprises a codon for each amino acid residue in the native Factor VIII:C polypeptide; and

b) modifying at least one codon that is adjacent to a codon encoding a non-activating Arg residue, to produce the nucleic acid molecule of claim 60.

80. The method of producing a nucleic acid molecule as claimed in claim 79, wherein the modifying is performed by site directed mutagenesis or by use of polymerase chain reaction techniques.

81. A method of producing a nucleic acid molecule that encodes a Factor VIII:C polypeptide analog comprising:

a) providing a nucleic acid molecule that encodes a native Factor VIII:C polypeptide, wherein the nucleic acid molecule comprises a codon for each amino acid residue in the native Factor VIII:C polypeptide; and

b) modifying at least one codon that is adjacent to a codon encoding a non-activating Arg residue, to produce the nucleic acid molecule of claim 61.

82. The method of producing a nucleic acid molecule as claimed in claim 81, wherein the modifying is performed by site directed mutagenesis or by use of polymerase chain reaction techniques.

83. A method of producing a recombinant vector that comprises a nucleic acid molecule that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising linking a regulatory element to the nucleic acid molecule of claim 59.

84. A method of producing a recombinant vector that comprises a nucleic acid molecule that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising linking a regulatory element to the nucleic acid molecule of claim 60.

85. A method of producing a recombinant vector that comprises a nucleic acid molecule that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising linking a regulatory element to the nucleic acid molecule of claim 61.

86. A method of producing a recombinant host cell that comprises a nucleic acid molecule that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising transforming a host cell with the nucleic acid molecule of claim 59.

87. A method of producing a recombinant host cell that comprises a nucleic acid molecule that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising transforming a host cell with the nucleic acid molecule of claim 60.

88. A method of producing a recombinant host cell that comprises a nucleic acid molecule that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising transforming a host cell with the nucleic acid molecule of claim 61.

89. A method of producing a recombinant host cell that comprises a recombinant vector that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising transforming a host cell with the recombinant vector of claim 62.

90. A method of producing a recombinant host cell that comprises a recombinant vector that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising transforming a host cell with the recombinant vector of claim 63.

91. A method of producing a recombinant host cell that comprises a recombinant vector that comprises a nucleotide sequence that encodes a Factor VIII:C polypeptide analog, comprising transforming a host cell with the recombinant vector of claim 64.

92. A pharmaceutical composition comprising the active Factor VIII:C polypeptide analog complex of claim 42 and a pharmaceutically acceptable excipient.

93. A pharmaceutical composition comprising the active Factor VIII:C polypeptide analog complex of claim 47 and a pharmaceutically acceptable excipient.

94. A pharmaceutical composition comprising the active Factor VIII:C polypeptide analog complex of claim 52 and a pharmaceutically acceptable excipient.

95. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the pharmaceutical composition of claim 92.

96. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising

administering thereto a therapeutically effective amount of the pharmaceutical composition of claim 93.

97. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the pharmaceutical composition of claim 94.

98. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 59.

99. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 60.

100. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 61.

101. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 62.

102. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 63.

103. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 64.

104. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 59 and an active Factor VIII:C polypeptide analog.

105. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 60 and an active Factor VIII:C polypeptide analog.

106. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 61 and an active Factor VIII:C polypeptide analog.

107. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 62 and an active Factor VIII:C polypeptide analog.

108. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 63 and an active Factor VIII:C polypeptide analog.

109. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 64 and an active Factor VIII:C polypeptide analog.

110. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 59 and an active Factor VIII:C analog complex.

111. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 60 and an active Factor VIII:C analog complex.

112. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the nucleic acid molecule of claim 61 and an active Factor VIII:C analog complex.

113. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 62 and an active Factor VIII:C analog complex.

114. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 63 and an active Factor VIII:C analog complex.

115. A method for prevention or treatment of active Factor VIII:C deficiency in a mammal comprising administering thereto a therapeutically effective amount of the recombinant vector of claim 64 and an active Factor VIII:C analog complex.

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                                -19                                 -10                                  1                                  10
                                Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys Leu Leu Arg Phe Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu
                                ATG CAA ATA GAG CTC TCC ACC TGC TTC TTT CTG TGC CTT TTG CGA TTC TGC TTT AGT GCC ACC AGA AGA TAC TAC CTG GGT GCA GTG GAA

                                        20                                  30                                  40
Leu Ser Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
CTG TCA TGG GAC TAT ATG CAA AGT GAT CTC GGT GAG CTG CCT GTG GAC GCA AGA TTT CCT CCT AGA GTG CCA AAA TCT TTT CCA TTC AAC ACC TCA GTC GTG TAC AAA AAG

    50                                  60                                  70                                  80
Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val Tyr Asp Thr Val Val
ACT CTG TTT GTA GAA TTC ACG GAT CAC CTT TTC AAC ATC GCT AAG CCA AGG CCA CCC TGG ATG GGT CTG CTA GGT CCT ACC ATC CAG GCT GAG GTT TAT GAT ACA GTG GTC

                90                                  100                                 110                                 120
Ile Thr Leu Lys Asn Met Ala Ser His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu
ATT ACA CTT AAG AAC ATG GCT TCC CAT CCT GTC AGT CTT CAT GCT GTT GGT GTA TCC TAC TGG AAA GCT TCT GAG GGA GCT GAA TAT GAT GAT CAG ACC AGT CAA AGG GAG

                130                                 140                                 150
Lys Glu Asp Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu
AAA GAA GAT GAT AAA GTC TTC CCT GGT GGA AGC CAT ACA TAT GTC TGG CAG GTC CTG AAA GAG AAT GGT CCA ATG GCC TCT GAC CCA CTG TGC CTT ACC TAC TCA TAT CTT

160                                 170                                 180                                 190
Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu His Lys Phe Ile
TCT CAT GTG GAC CTG GTA AAA GAC TTG AAT TCA GGC CTC ATT GGA GCC CTA CTA GTA TGT AGA GAA GGG AGT CTG GCC AAG GAA AAG ACA CAG ACC TTG CAC AAA TTT ATA

            200                                 210                                 220                                 230
Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr
CTA CTT TTT GCT GTA TTT GAT GAA GGG AAA AGT TGG CAC TCA GAA ACA AAG AAC TCC TTG ATG CAG GAT AGG GAT GCT GCA TCT GCT CGG GCC TGG CCT AAA ATG CAC ACA

                240                                 250                                 260                                 270
Val Asn Gly Tyr Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe
GTC AAT GGT TAT GTA AAC AGG TCT CTG CCA GGT CTG ATT GGA TGC CAC AGG AAA TCA GTC TAT TGG CAT GTG ATT GGA ATG GGC ACC ACT CCT GAA GTG CAC TCA ATA TTC

                280                                 290                                 300
Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly Gln Phe Leu
CTC GAA GGT CAC ACA TTT CTT GTG AGG AAC CAT CGC CAG GCG TCC TTG GAA ATC TCG CCA ATA ACT TTC CTT ACT GCT CAA ACA CTC TTG ATG GAC CTT GGA CAG TTT CTA

        310                                 320                                 330                                 340
Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu
CTG TTT TGT CAT ATC TCT TCC CAC CAA CAT GAT GGC ATG GAA GCT TAT GTC AAA GTA GAC AGC TGT CCA GAG GAA CCC CAA CTA CGA ATG AAA AAT AAT GAA GAA GCG GAA

                350                                 360                                 370                                 380
Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr
GAC TAT GAT GAT GAT CTT ACT GAT TCT GAA ATG GAT GTG GTC AGG TTT GAT GAT GAC AAC TCT CCT TCC TTT ATC CAA ATT CGC TCA GTT GCC AAG AAG CAT CCT AAA ACT

                390                                 400                                 410
Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
TGG GTA CAT TAC ATT GCT GCT GAA GAG GAG GAC TGG GAC TAT GCT CCC TTA GTC CTC GCC CCC GAT GAC AGA AGT TAT AAA AGT CAA TAT TTG AAC AAT GGC CCT CAG CGG
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Fig. 1A

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    420                                 430                                 440                                 450
Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly
ATT GGT AGG AAG TAC AAA AAA GTC CGA TTT ATG GCA TAC ACA GAT GAA ACC TTT AAG ACT CGT GAA GCT ATT CAG CAT GAA TCA GGA ATC TTG GGA CCT TTA CTT TAT GGG

            460                                 470                                 480                                 490
Glu Val Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro
GAA GTT GGA GAC ACA CTG TTG ATT ATA TTT AAG AAT CAA GCA AGC AGA CCA TAT AAC ATC TAC CCT CAC GGA ATC ACT GAT GTC CGT CCT TTG TAT TCA AGG AGA TTA CCA

                        500                                 510                                 520
Lys Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys Leu
AAA GGT GTA AAA CAT TTG AAG GAT TTT CCA ATT CTG CCA GGA GAA ATA TTC AAA TAT AAA TGG ACA GTG ACT GTA GAA GAT GGG CCA ACT AAA TCA GAT CCT CGG TGC CTG

530                                 540                                 550                                 560
Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp Gln Arg Gly Asn Gln Ile
ACC CGC TAT TAC TCT AGT TTC GTT AAT ATG GAG AGA GAT CTA GCT TCA GGA CTC ATT GGC CCT CTC CTC ATC TGC TAC AAA GAA TCT GTA GAT CAA AGA GGA AAC CAG ATA

        570                                 580                                 590                                 600
Met Ser Asp Lys Arg Asn Val Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu
ATG TCA GAC AAG AGG AAT GTC ATC CTG TTT TCT GTA TTT GAT GAG AAC CGA AGC TGG TAC CTC ACA GAG AAT ATA CAA CGC TTT CTC CCC AAT CCA GCT GGA GTG CAG CTT

                    610                                 620                                 630                                 640
Glu Asp Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
GAG GAT CCA GAG TTC CAA GCC TCC AAC ATC ATG CAC AGC ATC AAT GGC TAT GTT TTT GAT AGT TTG CAG TTG TCA GTT TGT TTG CAT GAG GTG GCA TAC TGG TAC ATT CTA

                            650                                 660                                 670
Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro Phe Ser Gly Glu Thr
AGC ATT GGA GCA CAG ACT GAC TTC CTT TCT GTC TTC TTC TCT GGA TAT ACC TTC AAA CAC AAA ATG GTC TAT GAA GAC ACA CTC ACC CTA TTC CCA TTC TCA GGA GAA ACT

    680                                 690                                 700                                 710
Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn
GTC TTC ATG TCG ATG GAA AAC CCA GGT CTA TGG ATT CTG GGG TGC CAC AAC TCA GAC TTT CGG AAC AGA GGC ATG ACC GCC TTA CTG AAG GTT TCT AGT TGT GAC AAG AAC

                720                                 730                                 740                                 750
Thr Gly Asp Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Thr
ACT GGT GAT TAT TAC GAG GAC AGT TAT GAA GAT ATT TCA GCA TAC TTG CTG AGT AAA AAC AAT GCC ATT GAA CCA AGA AGC TTC TCC CAG AAT TCA AGA CAC CCT AGC ACT

                        760                                 770                                 780
Arg Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn Val Ser Ser Ser
AGG CAA AAG CAA TTT AAT GCC ACC ACA ATT CCA GAA AAT GAC ATA GAG AAG ACT GAC CCT TGG TTT GCA CAC AGA ACA CCT ATG CCT AAA ATA CAA AAT GTC TCC TCT AGT
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Fig. 1B

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    790                                 800                                 810                                 820
Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile
GAT TTG TTG ATG CTC TTG CGA CAG AGT CCT ACT CCA CAT GGG CTA TCC TTA TCT GAT CTC CAA GAA GCC AAA TAT GAG ACT TTT TCT GAT GAT CCA TCA CCT GGA GCA ATA

            830                                 840                                 850                                 860
Asp Ser Asn Asn Ser Leu Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys
GAC AGT AAT AAC AGC CTG TCT GAA ATG ACA CAC TTC AGG CCA CAG CTC CAT CAC AGT GGG GAC ATG GTA TTT ACC CCT GAG TCA GGC CTC CAA TTA AGA TTA AAT GAG AAA

                        870                                 880                                 890
Leu Gly Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala Gly Thr Asp
CTG GGG ACA ACT GCA GCA ACA GAG TTG AAG AAA CTT GAT TTC AAA GTT TCT AGT ACA TCA AAT AAT CTG ATT TCA ACA ATT CCA TCA GAC AAT TTG GCA GCA GGT ACT GAT

900                 910                                 920                                 930
Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro Leu Thr Glu Ser Gly Gly Pro Leu
AAT ACA AGT TCC TTA GGA CCC CCA AGT ATG CCA GTT CAT TAT GAT AGT CAA TTA GAT ACC ACT CTA TTT GGC AAA AAG TCA TCT CCC CTT ACT GAG TCT GGT GGA CCT CTG

        940                                 950                                 960                                 970
Ser Leu Ser Glu Glu Asn Asn Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe
AGC TTG AGT GAA GAA AAT AAT GAT TCA AAG TTG TTA GAA TCA GGT TTA ATG AAT AGC CAA GAA AGT TCA TGG GGA AAA AAT GTA TCG TCA ACA GAG AGT GGT AGG TTA TTT

                    980                                 990                                 1000                                1010
Lys Gly Lys Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala Thr Asn
AAA GGG AAA AGA GCT CAT GGA CCT GCT TTG TTG ACT AAA GAT AAT GCC TTA TTC AAA GTT AGC ATC TCT TTG TTA AAG ACA AAC AAA ACT TCC AAT AAT TCA GCA ACT AAT

                            1020                                1030                                1040
Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu Phe Lys Lys Val Thr Pro Leu Ile His
AGA AAG ACT CAC ATT GAT GGC CCA TCA TTA TTA ATT GAG AAT AGT CCA TCA GTC TGG CAA AAT ATA TTA GAA AGT GAC ACT GAG TTT AAA AAA GTG ACA CCT TTG ATT CAT

    1050                                1060                                1070                                1080
Asp Arg Met Leu Met Asp Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly Pro
GAC AGA ATG CTT ATG GAC AAA AAT GCT ACA GCT TTG AGG CTA AAT CAT ATG TCA AAT AAA ACT ACT TCA TCA AAA AAC ATG GAA ATG GTC CAA CAG AAA AAA GAG GGC CCC

                1090                                1100                                1110                                1120
Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His Gly Lys Asn Ser Leu Asn Ser Gly
ATT CCA CCA GAT GCA CAA AAT CCA GAT ATG TCG TTC TTT AAG ATG CTA TTC TTG CCA GAA TCA GCA AGG TGG ATA CAA AGG ACT CAT GGA AAG AAC TCT CTG AAC TCT GGG

                        1130                                1140                                1150
Gln Gly Pro Ser Pro Lys Gln Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly Gln Asn Phe Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly Glu Phe Thr
CAA GGC CCC AGT CCA AAG CAA TTA GTA TCC TTA GGA CCA GAA AAA TCT GTG GAA GGT CAG AAT TTC TTG TCT GAG AAA AAC AAA GTG GTA GTA GGA AAG GGT GAA TTT ACA

  1160                                1170                                1180                                1190
Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln
AAG GAC GTA GGA CTC AAA GAG ATG GTT TTT CCA AGC AGC AGA AAC CTA TTT CTT ACT AAC TTG GAT AAT TTA CAT GAA AAT AAT ACA CAC AAT CAA GAA AAA AAA ATT CAG

            1200                                1210                                1220                                1230
Glu Glu Ile Glu Lys Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr
GAA GAA ATA GAA AAG AAG GAA ACA TTA ATC CAA GAG AAT GTA GTT TTG CCT CAG ATA CAT ACA GTG ACT GGC ACT AAG AAT TTC ATG AAG AAC CTT TTC TTA CTG AGC ACT
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Fig.1C

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                                1240                                1250                                1260
Arg Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys His Thr Ala His Phe Ser
AGG CAA AAT GTA GAA GGT TCA TAT GAC GGG GCA TAT GCT CCA GTA CTT CAA GAT TTT AGG TCA TTA AAT GAT TCA ACA AAT AGA ACA AAG AAA CAC ACA GCT CAT TTC TCA

1270                                1280                                1290                                1300
Lys Lys Gly Glu Glu Glu Asn Leu Glu Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe
AAA AAA GGG GAG GAA GAA AAC TTG GAA GGC TTG GGA AAT CAA ACC AAG CAA ATT GTA GAG AAA TAT GCA TGC ACC ACA AGG ATA TCT CCT AAT ACA AGC CAG CAG AAT TTT

        1310                                1320                                1330                                1340
Val Thr Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr Gln Trp Ser Lys Asn
GTC ACG CAA CGT AGT AAG AGA GCT TTG AAA CAA TTC AGA CTC CCA CTA GAA GAA ACA GAA CTT GAA AAA AGG ATA ATT GTG GAT GAC ACC TCA ACC CAG TGG TCC AAA AAC

                        1350                                1360                                1370                                1380
Met Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile
ATG AAA CAT TTG ACC CCG AGC ACC CTC ACA CAG ATA GAC TAC AAT GAG AAG GAG AAA GGG GCC ATT ACT CAG TCT CCC TTA TCA GAT TGC CTT ACG AGG AGT CAT AGC ATC

                                1390                                1400                                1410
Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe Gln Asp Asn Ser Ser His Leu Pro
CCT CAA GCA AAT AGA TCT CCA TTA CCC ATT GCA AAG GTA TCA TCA TTT CCA TCT ATT AGA CCT ATA TAT CTG ACC AGG GTC CTA TTC CAA GAC AAC TCT TCT CAT CTT CCA

        1420                                1430                                1440                                1450
Ala Ala Ser Tyr Arg Lys Lys Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly
GCA GCA TCT TAT AGA AAG AAA GAT TCT GGG GTC CAA GAA AGC AGT CAT TTC TTA CAA GGA GCC AAA AAA AAT AAC CTT TCT TTA GCC ATT CTA ACC TTG GAG ATG ACT GGT

                1460                                1470                                1480                                1490
Asp Gln Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr Ser Gly Lys
GAT CAA AGA GAG GTT GGC TCC CTG GGG ACA AGT GCC ACA AAT TCA GTC ACA TAC AAG AAA GTT GAG AAC ACT GTT CTC CCG AAA CCA GAC TTG CCC AAA ACA TCT GGC AAA

                        1500                                1510                                1520
Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys Asp Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His Leu Asp Leu Val Glu Gly Ser Leu Leu Gln Gly
GTT GAA TTG CTT CCA AAA GTT CAC ATT TAT CAG AAG GAC CTA TTC CCT ACG GAA ACT AGC AAT GGG TCT CCT GGC CAT CTG GAT CTC GTG GAA GGG AGC CTT CTT CAG GGA

    1530                                1540                                1550                                1560
Thr Glu Gly Ala Ile Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg Val Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp Pro Leu
ACA GAG GGA GCG ATT AAG TGG AAT GAA GCA AAC AGA CCT GGA AAA GTT CCC TTT CTG AGA GTA GCA ACA GAA AGC TCT GCA AAG ACT CCC TCC AAG CTA TTG GAT CCT CTT

                1570                                1580                                1590                                1600
Ala Trp Asp Asn His Tyr Gly Thr Gln Ile Pro Lys Glu Glu Trp Lys Ser Gln Glu Lys Ser Pro Glu Lys Thr Ala Phe Lys Lys Lys Asp Thr Ile Leu Ser Leu Asn
GCT TGG GAT AAC CAC TAT GGT ACT CAG ATA CCA AAA GAA GAG TGG AAA TCC CAA GAG AAG TCA CCA GAA AAA ACA GCT TTT AAG AAA AAG GAT ACC ATT TTG TCC CTG AAC

                        1610                                1620                                1630
Ala Cys Glu Ser Asn His Ala Ile Ala Ala Ile Asn Glu Gly Gln Asn Lys Pro Glu Ile Glu Val Thr Trp Ala Lys Gln Gly Arg Thr Glu Arg Leu Cys Ser Gln Asn
GCT TGT GAA AGC AAT CAT GCA ATA GCA GCA ATA AAT GAG GGA CAA AAT AAG CCC GAA ATA GAA GTC ACC TGG GCA AAG CAA GGT AGG ACT GAA AGG CTG TGC TCT CAA AAC

1640                                1650                                1660                                1670
Pro Pro Val Leu Lys Arg His Gln Arg Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp
CCA CCA GTC TTG AAA CGC CAT CAA CGG GAA ATA ACT CGT ACT ACT CTT CAG TCA GAT CAA GAG GAA ATT GAC TAT GAT GAT ACC ATA TCA GTT GAA ATG AAG AAG GAA GAT
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Fig.1D

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          1680                                1690                                1700                                1710
Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser
TTT GAC ATT TAT GAT GAG GAT GAA AAT CAG AGC CCC CGC AGC TTT CAA AAG AAA ACA CGA CAC TAT TTT ATT GCT GCA GTG GAG AGG CTC TGG GAT TAT GGG ATG AGT AGC

                    1720                                1730                                1740                                1750
Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly
TCC CCA CAT GTT CTA AGA AAC AGG GCT CAG AGT GGC AGT GTC CCT CAG TTC AAG AAA GTT GTT TTC CAG GAA TTT ACT GAT GGC TCC TTT ACT CAG CCC TTA TAC CGT GGA

                              1760                                1770                                1780
Glu Leu Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser
GAA CTA AAT GAA CAT TTG GGA CTC CTG GGG CCA TAT ATA AGA GCA GAA GTT GAA GAT AAT ATC ATG GTA ACT TTC AGA AAT CAG GCC TCT CGT CCC TAT TCC TTC TAT TCT

      1790                                1800                                1810                                1820
Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala
AGC CTT ATT TCT TAT GAG GAA GAT CAG AGG CAA GGA GCA GAA CCT AGA AAA AAC TTT GTC AAG CCT AAT GAA ACC AAA ACT TAC TTT TGG AAA GTG CAA CAT CAT ATG GCA

                    1830                                1840                                1850                                1860
Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn
CCC ACT AAA GAT GAG TTT GAC TGC AAA GCC TGG GCT TAT TTC TCT GAT GTT GAC CTG GAA AAA GAT GTG CAC TCA GGC CTG ATT GGA CCC CTT CTG GTC TGC CAC ACT AAC

                              1870                                1880                                1890
Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn
ACA CTG AAC CCT GCT CAT GGG AGA CAA GTG ACA GTA CAG GAA TTT GCT CTG TTT TTC ACC ATC TTT GAT GAG ACC AAA AGC TGG TAC TTC ACT GAA AAT ATG GAA AGA AAC

   1900                                1910                                1920                                1930
Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala
TGC AGG GCT CCC TGC AAT ATC CAG ATG GAA GAT CCC ACT TTT AAA GAG AAT TAT CGC TTC CAT GCA ATC AAT GGC TAC ATA ATG GAT ACA CTA CCT GGC TTA GTA ATG GCT

                1940                                1950                                1960                                1970
Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys Glu Glu Tyr Lys
CAG GAT CAA AGG ATT CGA TGG TAT CTG CTC AGC ATG GGC AGC AAT GAA AAC ATC CAT TCT ATT CAT TTC AGT GGA CAT GTG TTC ACT GTA CGA AAA AAA GAG GAG TAT AAA

                          1980                                1990                                2000
Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly
ATG GCA CTG TAC AAT CTC TAT CCA GGT GTT TTT GAG ACA GTG GAA ATG TTA CCA TCC AAA GCT GGA ATT TGG CGG GTG GAA TGC CTT ATT GGC GAG CAT CTA CAT GCT GGG

2010                                2020                                2030                                2040
Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp
ATG AGC ACA CTT TTT CTG GTG TAC AGC AAT AAG TGT CAG ACT CCC CTG GGA ATG GCT TCT GGA CAC ATT AGA GAT TTT CAG ATT ACA GCT TCA GGA CAA TAT GGA CAG TGG

          2050                                2060                                2070                                2080
Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly
GCC CCA AAG CTG GCC AGA CTT CAT TAT TCC GGA TCA ATC AAT GCC TGG AGC ACC AAG GAG CCC TTT TCT TGG ATC AAG GTG GAT CTG TTG GCA CCA ATG ATT ATT CAC GGC

                    2090                                2100                                2110                                2120
Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
ATC AAG ACC CAG GGT GCC CGT CAG AAG TTC TCC AGC CTC TAC ATC TCT CAG TTT ATC ATC ATG TAT AGT CTT GAT GGG AAG AAG TGG CAG ACT TAT CGA GGA AAT TCC ACT
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Fig.1E

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                                    2130                                    2140                                    2150
Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser
GGA ACC TTA ATG GTC TTC TTT GGC AAT GTG GAT TCA TCT GGG ATA AAA CAC AAT ATT TTT AAC CCT CCA ATT ATT GCT CGA TAC ATC CGT TTG CAC CCA ACT CAT TAT AGC

      2160                                    2170                                    2180                                    2190
Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser Ser
ATT CGC AGC ACT CTT CGC ATG GAG TTG ATG GGC TGT GAT TTA AAT AGT TGC AGC ATG CCA TTG GGA ATG GAG AGT AAA GCA ATA TCA GAT GCA CAG ATT ACT GCT TCA TCC

                2200                                    2210                                    2220                                    2230
Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln
TAC TTT ACC AAT ATG TTT GCC ACC TGG TCT CCT TCA AAA GCT CGA CTT CAC CTC CAA GGG AGG AGT AAT GCC TGG AGA CCT CAG GTG AAT AAT CCA AAA GAG TGG CTG CAA

                                2240                                    2250                                    2260
Val Asp Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly
GTG GAC TTC CAG AAG ACA ATG AAA GTC ACA GGA GTA ACT ACT CAG GGA GTA AAA TCT CTG CTT ACC AGC ATG TAT GTG AAG GAG TTC CTC ATC TCC AGC AGT CAA GAT GGC

   2270                                    2280                                    2290                                    2300
His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
CAT CAG TGG ACT CTC TTT TTT CAG AAT GGC AAA GTA AAG GTT TTT CAG GGA AAT CAA GAC TCC TTC ACA CCT GTG GTG AAC TCT CTA GAC CCA CCG TTA CTG ACT CGC TAC

                2310                                    2320                                    2330    2332
Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
CTT CGA ATT CAC CCC CAG AGT TGG GTG CAC CAG ATT GCC CTG AGG ATG GAG GTT CTG GGC TGC GAG GCA CAG GAC CTC TAC
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Fig. 1F

Inte tional Application No
PCT/US 96/11444

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/755 A61K38/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 309 237 (GENENTECH INC) 29 March 1989 see page 3, line 35 - line 51 see page 15 - page 16; example 7	39
A	WO,A,87 07144 (GENETICS INST) 3 December 1987 cited in the application see page 3, paragraph 2 - page 4, paragraph 1 see page 11 - page 12 see page 17 - page 18	
A	WO,A,88 08035 (GENETICS INST) 20 October 1988 see page 4, paragraph 3 - page 5, paragraph 1	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 November 1996	17.12.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/ 11444

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 95-115
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 95-115 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0309237	29-03-89	US-A-	5024939	18-06-91
		AT-T-	111156	15-09-94
		DE-D-	3851399	13-10-94
		DE-T-	3851399	20-04-95
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